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THE ANALYSIS OF A DOPA DECARBOXYLASE ACTIVITY VARIANT IN *DROSOPHILA MELANOGASTER*

by

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A THESIS

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Abstract

A collection of 109 wild-type *Drosophila* strains was surveyed for dopa decarboxylase (DDC) activity. Four strains representing both extremes of the range of activities were selected for further study. Genetic analysis showed that in three of these strains the genetic element(s) responsible for variation in DDC activity was located on the second chromosome. Further genetic experiments indicated that for one strain, WGM-40, activity variation segregated as a single unit. The results were consistent with a chromosomal location in the immediate vicinity of the structural gene for DDC. The element responsible for activity variation in WGM-40 was named *Ddc*$^{+4}$.

An examination of a *Ddc*$^{+4}$ strain throughout development revealed that the element was stage specific in it's effect. In early first instar larvae and in newly eclosed adults the *Ddc*$^{+4}$ strain had more activity than a Canton-S strain while at pupariation the variant had less activity. These activity differences were coincident with similar differences in the amount of DDC protein measured immunologically and in the size of the DDC transcript pool measured using northern analysis. These results imply that *Ddc*$^{+4}$ exerts it's effect either at transcription or during processing and transport of DDC transcripts.

The DNA in the *Ddc* region of *Ddc*$^{+4}$ was compared to that of Canton-S by examination of genomic Southern blots. In addition, the *Ddc* region from a *Ddc*$^{+4}$ strain was cloned in a

bacteriophage λ vector and compared to a cloned Canton-S *Ddc* region. Extensive restriction fragment size heterogeneity was discovered in a 10 Kb region including the *Ddc* gene. A total of five small insertions and one deletion were found in *Ddc*$^{+4}$ DNA relative to Canton-S. These differences lie in transcribed but noncoding regions of the *Ddc* gene as well as in 5' flanking regions. Extensive heterogeneity of this type has not been described in other between strain comparisons of DNA sequence.

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I. Introduction

Each cell type in a multicellular organism expresses a particular subset of the coding capacity of the genome. This is reflected in differences in the spectrum of proteins and/or poly-A$^+$ RNA monitored either before and after differentiation or between different terminally differentiated cell types (Affara et al., 1980; Bernstein and Donady, 1980; Biessman, 1981; Davidson and Britten, 1979; Sakoyama and Okubo, 1981). In addition, a cell or tissue may experience transient changes in the pattern of gene expression in response to external stimuli such as hormones (O'Malley and Means, 1974) or heat shock (Ashburner and Bonner, 1979).

During the last ten years the processes by which information flows from DNA sequence to phenotype have been found to be startlingly complex. Because of this complexity and because of the variety of regulatory mechanisms described to date, it is difficult to make any general statements about gene regulation.

In several systems, the regulation of gene expression has been shown to be at the transcriptional level. Pulse labelling of nuclear RNA generated direct evidence for transcriptional control in adenovirus (Darnell, 1982), SV 40 (Rio et al., 1980), and chicken globin genes (Weintraub et al., 1981). For many other genes, in a variety of organsims, the examination of transcriptional control, has by necessity been less direct. Usually changes in the pool size of a

particular transcript are taken as reasonable indicators of transcriptional modulation. However, it is important to recognize the potential for additional regulatory events between RNA polymerase activity and changes in transcript pool size.

Apart from quantitative differences in the level of transcription, two examples have been described of tissue specific differences in the site of transcription initiation. Transcription of the α-amylase gene in mouse starts at different DNA sites in the liver and salivary gland (Hagenbuchle et al., 1981). In *Drosophila*, Benyajati et al. (1983) have shown that there are two different promoters for the structural gene for alcohol dehydrogenase. One promoter is active in larvae and the other, which lies 650 nucleotides upstream, is active in adults. Most of the long 5' leader sequence in the adult transcript is spliced out, yielding a mature mRNA of about the same size as the larval mRNA.

Once the primary transcript has been produced, a series of events jointly called RNA processing occurs. During processing noncoding intervening sequences are removed from the transcript by splicing, a poly-A tail is added to the 3' end of the mRNA, and the message is transported through the nuclear membrane and into the cytoplasm where it can be translated. Regulation of gene expression can potentially occur at any point during RNA processing and transport. In the rat calcitonin gene (Amara et al., 1982) and the mouse

αA-crystallin gene (King and Piatigorsky, 1983) differential splicing yields two distinctly different mRNA species. It is not clear that these two examples of differential splicing have regulatory significance. On the other hand, changes in splicing pattern and the selection of the polyadenylation site are critical in adenovirus. As virus infection proceeds different polyadenylation sites are selected on the same long transcript. This is associated with differences in splicing and results in the generation of different mRNA species (Nevins and Darnell, 1978).

The stability of mRNA may be an important parameter in regulation of gene expression. Chung et al.(1981) have described differences in mRNA stabilities associated with differentiation of the slime mold *Dictyostelium*. Additional regulation may take place at the level of translation. For example, in *Drosophila* the heat shock response involves preferential translation of heat shock specific mRNAs (Ballinger and Pardue, 1983).

Traditionally, the geneticist's approach to studying gene regulation has been to identify genetic elements which perturb or affect the expression of a particular gene. It has been convenient to classify such regulatory elements as being either cis-acting or trans-acting. A trans-acting element does not need to be colinear with the regulated gene in order to affect it. These elements may act at any stage in the path between gene and protein. The lack of any specific expectation regarding the action of trans-acting

elements makes their study difficult, and hence not particularly attractive. Cis-acting elements on the other hand are situated adjacent to the regulated gene and may, but do not necessarily, identify transcriptional signals. This last caveat is important in the light of the regulatory complexity described above.

A. Genetic analysis of trans-acting regulatory elements

The expression of a particular gene can depend a great deal on the genetic background in which it finds itself. Usually geneticists take care to exclude variation in gene expression due to background differences. However, several researchers have systematically searched for and analyzed genes which affect the expression of other genes from a distance and in a trans-acting way.

Scandalios et al., (1980) report work on a trans-acting modifier of catalyase activity in maize. Their evidence suggests that this putative regulatory gene called, *car-1*, acts at the level of enzyme synthesis. They were not able to distinguish between a transcriptional, RNA processing, or translational effect.

Abraham and Doane (1978) have characterized a locus called *map* in *D. melanogaster* that modifies the expression of the structural gene for α-amylase. The two genes are both on the second chromosome but are separated by 2 map units. Different alleles of *map* have different tissue specific patterns of α-amylase activity.

In a more extensive study Laurie-Ahlberg and collaborators (Laurie-Ahlberg et al., 1982; Wilton et al., 1982) have carefully described the sources of variation for 23 metabolic enzymes in *D. melanogaster*. They use isogenic second and third chromosome substitution lines to identify trans-acting genetic sources of variation for many of these enzymes. Additionally, they demonstrate some correlation in activity among groups of enzymes with related metabolic functions. This suggests that some of the variation observed may be due to genetic elements which affect, and possibly coordinately regulate related enzymes.

The manner in which these trans-acting elements influence expression of distant genes is unknown. However, studies on the yeast mating-type system have shown how some elements might act. Mating-type is determined by a single locus, *MAT*, for which there are two alleles, *MATa* and *MAT*α. Silent copies of *MATa* and *MAT*α, called *HMRa* and *HML*α respectively, flank the functioning *MAT* locus (Strathern et al., 1980). These silent copies have the same DNA sequence as their active counterparts at *MAT*. Repression of *HMRa* and *HML*α is mediated by several unlinked genes, one of which is called *SIR* (Nasmyth, 1982). Mutations at *SIR* cause the silent copies to be expressed and at the same time cause changes in the chromatin around *HMRa* and *HML*α. Nasmyth (1982) speculates that the *SIR* gene product is responsible for maintaining the chromatin around *HMRa* and *HML*α in a configuration that prevents expression.

B. Genetic analysis of cis-acting regulatory elements

Despite the obvious differences in complexity between gene expression in prokaryotes and eukaryotes, some similarities in the mechanism of transcription probably exist. The prokaryotic promoter is built around a pair of conserved sequences positioned 10 nucleotides and 35 nucleotides upstream from the transcription start site. These sequences are called the Pribnow box and appear to be the basic sequence requirements for *Escherichia coli* RNA polymerase binding and transcription initiation. Associated with this basic promoter are other sequences, called operator sequences, which mediate transcriptional control of the operon by the cell. These sequences usually vary considerably from operon to operon. Our naive expection is that something similar to these two kinds of sequences will be present adjacent to eukaryotic protein coding genes as well.

In prokaryotes, both the operator and the promoter were defined genetically by the imaginative use of mutants. In eukaryotes, classical mutagenesis and genetic analysis has been less successful. This is partly because, with a few exceptions, there is a lack of powerful selection procedures. However this does not seem to be the whole story. For example Sherman et al., (1981) used strong selection to look for mutants which overproduce iso-2-cytochrome c in the yeast *Saccharomyces cerevisiae*. They found overproducers only rarely and those they did find

contained chromosomal rearrangements 5' to the iso-2-cytochrome c gene. From this evidence they suggest that eukaryotic regulatory regions are generally not mutable by single base pair changes. Similarly, Fink and collaborators (Donahue et al., 1982) have been unable to isolate single base pair mutations in the 5' noncoding region of the yeast *His 4* gene.

The inability to recover regulatory mutants from mutagenized populations has prompted researchers to use natural populations as a source of regulatory variation. Briefly, the approach has been to first identify variation at the enzyme activity level, and then try to demonstrate in a variety of ways, that the observed variation is not due to structural changes in the enzyme. The focus is on transcriptional control, although this is not always explicitly stated.

The xanthine dehydrogenase (XDH) gene-enzyme system in *Drosophila* has been particularly amenable to this kind of analysis for two reasons. Firstly, larvae lacking XDH die when purine is included in their food. Positive selection for purine resistant larvae facilitated sophisticated fine structure mapping of the XDH structural locus. Secondly, electrophoretic variants can be used as markers to determine if regulatory elements are cis-acting. Chovnick and his group (McCarron et al., 1979) have identified two XDH activity variants; one underproducer and one overproducer. The underproducer has about half and the overproducer about

twice the activity of their standard strain. The elements responsible for both these activity differences map to the left of the leftmost structural allele. Both are cis-acting and studies with anti-XDH antiserum show that the amount of cross-reacting material correlates with enzyme activity. Chovnick therefore suggests (McCarron et al., 1979), that these two elements define a control element that lies adjacent to the structural gene for XDH.

Natural variation in enzyme activity has also been carefully analyzed for β-glucuronidase in mice (Paigen, 1979). The appearance of this enzyme in mouse kidney cells is induced by androgens. Different mouse strains were found to induce β-glucuronidase activity to varying extents. Genetic analysis identified an element called *Gus-r* which is responsible for this variation. This element is closely linked to the structural gene, *Gus-s*, and is cis-acting. Variation at *Gus-r* has no effect on the basal level of β-glucuronidase in kidney or the level of enzyme in other tissues. More recently Paigen's group has shown that *Gus-r* affects the synthesis of mRNA for β-glucuronidase after administration of androgen (Watson et al., 1981). They hypothesize that *Gus-r* defines the chromatin region that binds the androgen-receptor complex thought to be involved in hormone mediated induction.

Further evidence for cis-acting regulators of gene expression is provided by the work of Dickinson on several gene-enzyme systems in various species of *Drosophila*.

Dickinson has isolated and mapped a putative regulatory element adjacent to the aldehyde oxidase (AO) gene in *D. melanogaster* (Dickinson, 1975). This element affects AO activity at pupariation only and hence is stage specific. In addition, Dickinson has worked on the genetic control of tissue specificity of gene expression. He found that the enzyme alcohol dehydrogenase (ADH) has different tissue specific patterns of expression in two closely related species of *Drosophila*; *D. grimshawi* and *D. orthofascia* (Dickinson and Carson, 1979). The enzyme also has a different electrophoretic mobility in these two species. When a *D. grimshawi* X *D. orthofascia* hybrid was created, each electrophoretic form of the enzyme was present only in those tissues characteristic of the parent species. This implies that cis-acting regulatory elements control tissue specific patterns of ADH expression. More recently it has been shown that these elements affect the amount of ADH mRNA in each tissue (Rabinow and Dickinson 1981).

As I stated earlier the focus of all of these studies is on transcriptional control, even though it is not always stated explicitly. A logical question therefore is: what are the chances of identifying variation in transcriptional control in material selected on the basis of variation in the amount of a protein? A large amount of information gathered on globin gene expression in humans addresses this question. If either α or β globin is synthesized at a reduced level, or is not synthesized at all, the result is a

disease of the blood cells called thalassaemia. The great majority of thalassaemia patients turn out to have cis-acting mutations in one of their globin genes (Weatherall and Clegg, 1982). Surprisingly, out of perhaps 20 to 30 mutant genes examined, only one has a mutation in a putative control region 5' to the gene (Orkin et al., 1982). This β-globin gene has a C to G transversion 87 nucleotides upstream from the transcription start site. Other studies have shown this region to be important for globin gene expression (see below). The rest of the mutants are very diverse at the molecular level. Quite a few thalassaemias are due to deletions of various sizes. Others are due to nonsense mutations. Members of one interesting class of β-thalassaemias all have mutations in intervening sequences. These mutations disrupt splicing and prevent production of normal amounts of mature mRNA (Treisman et al., 1983). Another β-thalassaemia is characterized by an unusually unstable mRNA (Maquat et al., 1981).

Except for the one C to G transversion mentioned above, none of these mutations affect true regulatory processes, at least as far as the globin genes are concerned. This is undoubtedly because there are many ways of disrupting gene function without affecting regulatory processes and a study of thalassaemia will involve only mutations causing loss of gene function. Studies involving complex regulatory variation, such as stage or tissue specific variation are of special interest for this reason.

C. Molecular studies of gene transcription

In the past several years the approaches described above have been complemented and in some ways supplanted by what has been called surrogate genetics. A large number of eukaryotic genes have now been cloned and sophisticated methods of introducing a variety of different mutations into these genes have been developed. Once constructed, there are several *in vitro* transcription systems in use for testing the effects of these mutations on transcription. Alternatively the mutant gene can be reintroduced into a cell by DNA-mediated transformation. Until recently transformation was possible with bacterial, yeast, and mammalian tissue culture cells only. However the list of transformable organisms is constantly growing, and now includes *Drosophila* (Rubin and Spradling, 1982).

The starting material for these kinds of studies is DNA sequence information. Hogness and Goldberg (Goldberg, 1979) noticed a region 25 to 30 nucleotide pairs upstream from the mRNA start site which is conserved in all polymerase II transcribed genes. The importance of this sequence, now known as the T-A-T-A box, has been examined for a number of different genes (Breathnach and Chambon, 1981; Hirose et al., 1982; Mathis and Chambon, 1981). Sequences upstream from the mRNA start site were altered *in vitro* and the DNA tested for it's ability to support transcription in one of several *in vitro* transcription systems. In every case the T-A-T-A box was shown to be essential for normal

transcription. Studies *in vivo* (Benoist and Chambon, 1981; Grosschedl and Birnstiel 1980; Grosschedl et al., 1981; Grosveld et al., 1982; McKnight and Kingsbury, 1982) also demonstrate the importance of the T-A-T-A box. However it seems that for some genes, for instance the *Herpes simplex* thymidine kinase gene (McKnight and Kingsbury, 1982), the loss of the T-A-T-A sequence affects both the site of initiation of transcription as well as the rate of transcription whereas in others, such as the SV 40 early transcription unit (Benoist and Chambon, 1981), this same loss affects only the choice of initiation site but not the actual rate of transcription. Chambon and his group find that the initiation site at the SV 40 early promotor can be moved further downstream from the normal start site by deleting sequences between the start site and the T-A-T-A box. In the deletion strains the distance from the T-A-T-A sequence to the site of initiation is always about 25 nucleotide pairs. This suggests that the T-A-T-A sequence is important for positioning the RNA polymerase for initiation of transcription. In a further study, (Davison et al., 1983) Chambon and collaborators demonstrate the specific binding of transcription factors to the T-A-T-A box. These factors are derived from HeLa cells and are essential for transcription in crude cellular extracts.

All of this evidence suggests that the T-A-T-A box is an important part of the RNA polymerase II promoter. However sequences further upstream also affect transcription. Hen et

al., (1982) find that sequences 34 to 97 nucleotides upstream from the mRNA start site are required for expression at the adenovirus major late promoter both *in vitro* and *in vivo*. Unfortunately, they find that expression of the adenovirus genes on their plasmid is also dependent upon the presence of a 72 nucleotide sequence from SV 40 called the enhancer sequence (see below). This sequence enhances transcription of many genes from a distance in some unknown way (Wasylyk et al., 1983). This requirement complicates the interpretation of their experiment. The sequences between nucleotides 34 and 97 may be important only when associated with enhancer sequences. McKnight and Kingsbury (1982) also report an upstream effect on transcription of the herpes simplex thymidine kinase gene. They find two sequence blocks, one 47 to 61 nucleotides upstream and the other 80 to 105 nucleotides upstream which are important for normal *in vivo* expression. In a similar way, sequences between 50 and 100 nucleotides upstream from the transcription start site of the rabbit β-globin gene are required for transcription (Grosveld et al., 1982). This is consistent with the discovery, mentioned earlier, of a β-thalassaemia with a single base change at the -87 position. This mutation was found to decrease the rate of transcription of the β-globin gene *in vivo* (Treisman et al., 1983).

It is possible that these upstream sequences are also part of the basic RNA polymerase II promoter, here defined

as the minimal sequence requirement for polymerase function. However sequence consensus is not as strong in these regions as it is for the T-A-T-A box and no clear pattern of effects has yet emerged from *in vitro* mutagenesis experiments. Perhaps some of these sequences are gene specific and represent regulatory sequences distinct from the basic promoter.

Support for this idea comes from studies in *Drosophila* and yeast. Hugh Pelham (1982) has been studying the expression of the Hsp 70 heat-shock gene from *Drosophila* by inserting this gene into a vector containing the SV 40 origin of replication, and transforming monkey cells that support high-level replication of the vector. He found that, as in *Drosophila*, expression of the Hsp 70 gene could be induced by heat shocking the monkey cells. Using a series of Hsp 70 genes with different small deletions in the 5' region he showed that induction depended on sequences 47 to 66 nucleotides upstream of the mRNA start site. A similar sequence is found in approximately the same location in several other *Drosophila* heat shock genes. Fink and collaborators have been studying expression of genes involved in amino acid biosynthesis in yeast (Donahue et al., 1983). The expression of genes in several amino acid biosynthetic pathways is regulated coordinately by what is called the general amino acid control system. Starvation for any one of these amino acids results in increased transcription of all the genes in the general control

system. Fink's group has shown that a 6 nucleotide sequence repeated several times in the 5' nontranscribed region of these genes is necessary for this derepression.

A special kind of upstream sequence exists 5' to the SV40 early promoter. This 72 nucleotide sequence, called the enhancer or activator (Wasylyk et al., 1983), is present as a tandem repeat starting 115 nucleotides upstream of the mRNA start site and extending upstream. Deletion of both members of the tandem repeat completely eliminates transcription from the early promoter. However transcription is restored if the element is replaced, irrespective of its orientation or exact position relative to the mRNA start site. This sequence also seems to enhance transcription at other eukaryotic polymerase II promoters and even activates transcription where no promoter is known to exist (Wasylyk et al., 1983). Again neither the orientation nor the exact position of the element with respect to the start site are critical. However the strength of the effect does decrease with distance. Similar sequences are found in the long terminal repeat (LTR) region of some animal RNA viruses. Levison et al., (1982) show that a 72 nucleotide tandemly repeated sequence from the LTR of Moloney sarcoma virus can function in SV 40 in place of the SV 40 tandem repeat. There is no sequence homology between these two sequences. Wasylyk et al., (1983) suggest that enhancer sequences act as a site for the entry of RNA polymerase II. The polymerase is then able to scan up and down the DNA in search of transcription

start signals, possibly the T-A-T-A box.

D. Chromatin structure and gene expression

Work on enhancer sequences indicates that quite different nucleotide sequences can have similar regulatory effects. This may be because a specific chromatin configuration, perhaps a configuration essential for gene expression, could be compatible with several primary sequence arrangements. However, this is sensible only if chromatin structure can be shown to affect gene expression. Chromatin, in it's most basic form, consists of DNA plus the 5 histone proteins organized in a repetitive array of nucleosome units. Further structural complexities may arise when this basic structure interacts with variant histone proteins and a diverse collection of non-histone proteins.

The first direct evidence that gene expression is accompanied by a change in chromatin structure came from work by Weintraub and Groudine (1976). They found that actively expressed globin genes are more sensitive to the endonuclease DNase I than are inactive globin genes. Further, they found that for chicken globin genes this increased sensitivity is dependent upon the presence of 2 specific non-histone proteins (Weisbrod and Weintraub, 1979).

Differential nuclease sensitivity has now been documented for a number of genes including the *Drosophila* heat shock genes (Wu et al., 1979), and histone genes (Samal

et al., 1981), the conalbumin gene in chickens (Kuo et al., 1979), and mating type loci in yeast (Nasmyth, 1982). In addition to a general increase in DNase I sensitivity these researchers find that specific sites of DNase I hypersensitivity are associated with actively expressed genes (Elgin, 1981). Hypersensitive sites are invariably but not exclusively present at the 5' end of actively expressed genes. In the α-globin gene of chickens the 5' hypersensitive site appears before the gene is expressed but only in tissues where expression will eventually occur. This means that the hypersensitive site is probably necessary but not sufficient for globin gene expression (Weintraub et al.,1981).

A close relationship between hypersensitive sites and gene expression is indicated by work on the *Sgs 4* gene in *Drosophila*. This gene codes for one of the glue proteins which are secreted by the salivary glands of the organism during pupariation. Shermoen and Beckendorf (1982) find that 5 hypersensitive sites appear 5' to the gene when it is expressed during late third instar. Muskavitch and Hogness (1982) studying variant strains which do not express this gene find that loss of gene expression correlates with loss of one or more of these hypersensitive sites. In another study (McGinnis et al., 1983) a single base change within a hypersensitive region correlates with a 50% reduction in *Sgs 4* specific mRNA.

The exact nature of DNase I hypersensitive sites has not been determined. There is some suggestion that they represent regions of single stranded DNA. Single strandedness implies an absence of nucleosomal organization. This may make the DNA more accessible and hence more sensitive to macromolecular probes such as nucleases (Larsen and Weintraub, 1982).

E. The Dopa decarboxylase gene-enzyme system

The enzyme Dopa decarboxylase (DDC) is a homodimer with subunit molecular weight of 54,000 daltons. It catalyzes the conversion of Dopa to dopamine (Clark et al., 1978). Dopamine in turn is required in epidermal cells for production of N-acetyldopamine, a compound involved in the sclerotization or hardening of newly deposited cuticle (Dickinson and Sullivan, 1975). In addition dopamine is thought to function as a neurotransmitter in neural tissue. Genetic evidence indicates that the same enzyme is responsible for dopamine production in both these tissues (Livingstone and Tempel, 1983; Wright et al., 1982). Enzyme activity also appears in ovarian tissue but the function of this activity is not known.

The gene coding for DDC was localized by recombinational analysis (Wright et al., 1976), to map position 54.1 on the left arm of chromosome 2. Since then numerous mutant alleles of the *Ddc* gene have been isolated, including null alleles, which behave as recessive lethals,

and two temperature sensitive alleles (Wright et al., 1981). More recently, a fragment of DNA containing the *Ddc* gene was isolated from a Charon 4A recombinant library (Hirsh and Davidson, 1981).

The expression of the *Ddc* gene is highly regulated both in a spatial and temporal sense. As noted above activity is present in 3 distinct tissues. However the majority of total DDC activity appears in the epidermis at 6 specific stages during the animals' life cycle. These stages are hatching, the 2 larval molts, pupariation, pupation, and adult eclosion (Kraminsky et al., 1980; Wright et al., 1982).

Early interest in the DDC system focussed on the induction of enzyme activity at pupariation. Experiments by Karlson and collaborators (Karlson and Sekeris, 1966) on *Calliphora* species showed that the steroid hormone ecdysterone was necessary for the appearance of DDC activity at pupariation. Fragoulis and Sekeris (1975a and 1975b) later demonstrated that the appearance of enzyme activity represented *de novo* synthesis of enzyme and that this synthesis was coincident with the appearance of translatable DDC mRNA. In *Drosophila* elegant experiments using the ecdysoneless mutant *edc*^{1} showed that ecdysterone induces a rapid increase in translatable DDC mRNA (Kraminsky et al., 1980). More recently Clark and Hodgetts (personal communication) have shown that the appearance of DDC transcript after ecdysterone treatment is independent of protein synthesis. Ashburner and Berendes (1978), working on

polytene chromosomes puffs in salivary glands also found that rapid responses to ecdysterone are independent of protein synthesis. Based on these observations they suggest that ecdysterone or an ecdysterone-receptor complex is directly affecting the rate of transcription.

Recently two studies have strengthened the hypothesis that steroid hormones, when associated with receptor proteins, interact directly with chromatin. Gronemeyer and Pongs (1980) localized ecdysterone-receptor complexes on polytene chromosomes by chemically crosslinking these complexes *in situ* and labelling with immunoflourescent techniques. They found that ecdysterone was situated at sites where hormone is known to induce puffing. Mulvihill et al., (1982) have identified a DNA sequence 250 to 300 nucleotides upstream from several progesterone responsive genes in chicken that seems to preferentially bind progesterone-receptor complexes. Hopefully a similar site will be identified for ecdysterone recognition when more ecdysterone-responsive genes are analyzed.

Unlike pupariation, the peaks of enzyme activity appearing at hatching, the 2 larval molts, and eclosion are not coincident with increases in ecdysterone titer. In addition there is probably *Ddc* gene expression in neural tissue throughout development. This suggests that the mechanism of *Ddc* gene regulation may be different in different stages and in different tissues. For example Gietz and Hodgetts (pers. comm.) have analyzed *Ddc* expression

during embryogenesis. They find that DDC transcript begins to accumulate late in embryogenesis just shortly before the peak of DDC activity. The ecdysterone titer at this time is very low.

The highly regulated pattern of gene expression described above makes the *Ddc* gene an ideal candidate for the study of gene regulation. My approach has been to identify and characterize contiguous genetic elements that modify the expression of this gene. As I mentioned earlier some studies suggest that conventional mutagenesis does not efficiently produce regulatory mutants. Natural populations represent an alternative source of regulatory variation. For this reason Dr. Ross Hodgetts conducted a screen of 109 strains isogenic for independently isolated second chromosomes. This screen identified the several strains of *Drosophila* with altered activity levels which represent the raw material for this work. One of these strains is analyzed here in detail and found to be stage-specific in its variation. Mapping experiments show that the element responsible for activity variation in this strain is closely linked to the structural gene for DDC. By using Southern blotting analysis and detailed restriction mapping of cloned DNA, several sequence polymorphisms were identified in the region of the *Ddc* gene in the variant strain. One or more of these polymorphisms may be responsible for the activity variation and therefore identify sequences important for the regulation of the *Ddc* gene.

II. Materials and Methods

A. Growth media and solutions

LB - The media used for growing all bacterial strains was Luria broth. This contained per liter: 10 gms Bacto-tryptone, 10 gms NaCl, and 5 gms Bacto-yeast extract. Solid media was LB containing 15 gm/l Bacto-agar. Media used to culture strains carrying plasmids conferring antibiotic resistance were supplemented with 20 mg/l tetracycline or 100 mg/l ampicillin. The antibiotics were added after the medium was autoclaved.

LMB - Bacterial cultures used for growth of bacteriophage λ were grown in LB plus 10mM $MgSO_4$.

TE - DNA preparations were typically stored in TE buffer which was 10mM Tris HCl (pH 7.5), 1mM EDTA.

TMG - Bacteriophage λ particles were handled and stored in TMG buffer. This buffer contained 10mM Tris HCl (pH 7.5), 10mM $MgSO_4$, 0.1% w/v gelatin.

SSC - Blotting procedures involved the use of a high salt solution called SSC. This solution contained 150mM NaCl, 15mM trisodium citrate.

Extraction buffer - Crude extracts of *Drosophila melanogaster* were prepared in extraction buffer containing 50mM Tris HCl (pH 7.3), 1mM phenylthiourea.

B. Stocks

All *Drosophila melanogaster* stocks were routinely maintained on a standard yeast-sucrose medium (Nash and Bell, 1968) at 25° C in complete darkness. Table 1 gives a complete list of all *D. melanogaster* stocks used and their origin. Table 2 provides the same information for the bacterial and bacteriophage stocks used in this study.

C. Determination of post embryonic DDC activity and ecdysterone titer

Growth and staging of organisms

Canton S embryos were collected at 25° C in a large population cage on 1.5% agar partially covered with live yeast paste. Time zero for each sample was taken to be the midpoint of a two hour laying period. The first collection of each day was discarded because these collections probably contained many withheld eggs. In order to maintain reasonable synchrony the organisms were staged at four times during development: hatch, the second instar to third instar molt, pupariation, and eclosion.

Staging at hatch

Approximately two hours before the expected mean hatch time the agar trays containing the embryos were washed gently with distilled water. All newly hatched first instar larvae were washed away while the partially embedded eggs

Table 1

Drosophila melanogaster Stock List

Stock	Source
109 wild-type lines	Drs. G. Bewley and G. Carmody
Cy/Pm ; Sb/Ubx	Dr. J.B. Bell
rdo hk pr/rdo hk pr	Dr. R.B. Hodgetts
b rdo hk pr cn/b rdo hk pr cn	This study
rdo hk pr/CyO†	Dr. R.B. Hodgetts
hk/hk	Bowling Green Stock Center
Ddc^{+4} *pr/Ddc*$^{+4}$ *pr*	This study
Canton-S	Dr. R.B. Hodgetts
Df(2L)130 *cn bw/CyO*†	Dr. R.B. Hodgetts

† *CyO* – In(2LR)*O*, *dp Cy pr cn*

Table 2

Bacterial and Bacteriophage Stocks

Strain	Markers	Source
E. coli		
HB 101	F$^-$, hsd S20(r¤m*), rec A13, ara-14, pro A2, lac Y1, gal k2, rps L20(Sm), xyl-5, mtl-1, supE44, λ$^-$	Dr. R.B. Hodgetts
Q 358	hsd R†, hsd M‡, supF, ø80	BRL
Q 359	hsd R†, hsd M‡, supF, ø80, P2	BRL
BHB 2688	N205 rec A$^-$[λimm^{434}, cIts, b2, red$^-$, Eam, Sam/λ]	Dr. R.Y.C. Lo
BHB 2690	N205 rec A$^-$[λimm^{434}, cIts, b2, red$^-$, Dam/λ]	Dr. R.Y.C. Lo
Bacteriophage λ		
λ1059	hλs Bam I° b189 (int29, NL44, CI857, pacI29) (int-cIII) KH54, SRI4°, NIN5, chi3	BRL
λCI 857	CI857	W.C. Clark

¤ - r_B^-

* - m_B^-

† - R_k^-

‡ - M_k^+

remained stuck in the agar. After a two hour wait the wash was repeated and the newly hatched larvae collected on a Nitex screen. These larvae were placed on agar completely covered with live yeast paste and incubated at 25° C. One more two hour collection was made for each tray.

Staging at the second instar to third instar molt

It is fairly easy to distinguish between second and third instar larvae by looking at the anterior spiracles. The anterior spiracles of second instar larvae are small and rudimentary when compared to those of third instar larvae (Bodenstein 1950). As a sample approached the time of second to third molt the larvae were removed from the live yeast paste by collection on a Nitex screen, washed with distilled water and placed on a moistened filter paper in a glass petri dish. By using a dissecting microscope third instar larvae were identified and removed from the sample. Two hours later third instar larvae were again removed and placed on agar covered with live yeast paste. This cycle was repeated two or three times.

Staging at pupariation

For a period of approximately one hour after pupariation the prepupae remain white. These white prepupae were picked from the sides of a tray and placed in a petri dish lined with moistened filter paper.

Staging at eclosion

Newly eclosed adults (0-2 hours) were collected by clearing bottles and collecting two hours later.

Assays

Samples were removed from the live yeast paste or filter paper at various times throughout postembryonic development and immediately frozen in liquid nitrogen. Extracts for enzyme assays were prepared at a concentration of 50 mg live weight per ml in extraction buffer using a motor driven teflon pestle in a glass homogenizer. The extracts were cleared by centrifugation at 27,000 x g for ten minutes. DDC assays were done according to Clark et al., (1978). Determination of protein concentration was done as described by Spector (1978).

Extracts to be used for the determination of ecdysterone titer were prepared by grinding larvae in 70% methanol, 1 mM phenythiourea at a concentration of 50 mgs/ml in a teflon grinder followed by centrifugation at 27,000 x g for ten minutes. The ecdysterone titers were determined by radioimmune assay in the laboratory of Dr. J.D. O'Connor at the Department of Biology, U.C.L.A. Hormone assays were done on larval samples only, so as to complement data published by Hodgetts et al., (1977).

D. Survey of DDC activity

Newly eclosed adults (0-2 hours old) were collected from each of 109 stocks isogenic for independently isolated wild second chromosomes. Extracts were made at a concentration of 20 mgs/ml in extraction buffer. Each extract was assayed for DDC activity in duplicate according to Clark et al., (1978).

E. Genetic analysis

In order to determine the second chromosome contribution to the activity variation observed, the crosses outlined in Figure 1 were performed. Newly eclosed F2 progeny of genotypes $Cy/Cy^{+};Sb^{+}/Sb^{+}$ and $Cy^{+}/Cy^{+};Sb/Sb^{+}$ were assayed for DDC activity. An equal number of male and female flies were included in each sample. If a strain showed activity variation segregating with the second chromosome the crosses described in Figure 2 were performed. These crosses were designed to show whether or not the element responsible for activity variation lies between *hk* and *pr* on the second chromosome, a region which includes the structural gene for DDC (Wright et al., 1976).

F. Immunotitration of DDC

The amount of cross reacting material (CRM) in a sample was estimated by determining the concentration of antiserum sufficient to precipitate half the DDC activity in that sample. Antiserum was prepared as described in Clark et al.,

Figure 1: Crossing scheme used to determine the autosomal segregation of DDC activity variation. All genetic markers are described in Lindsley and Grell (1967).

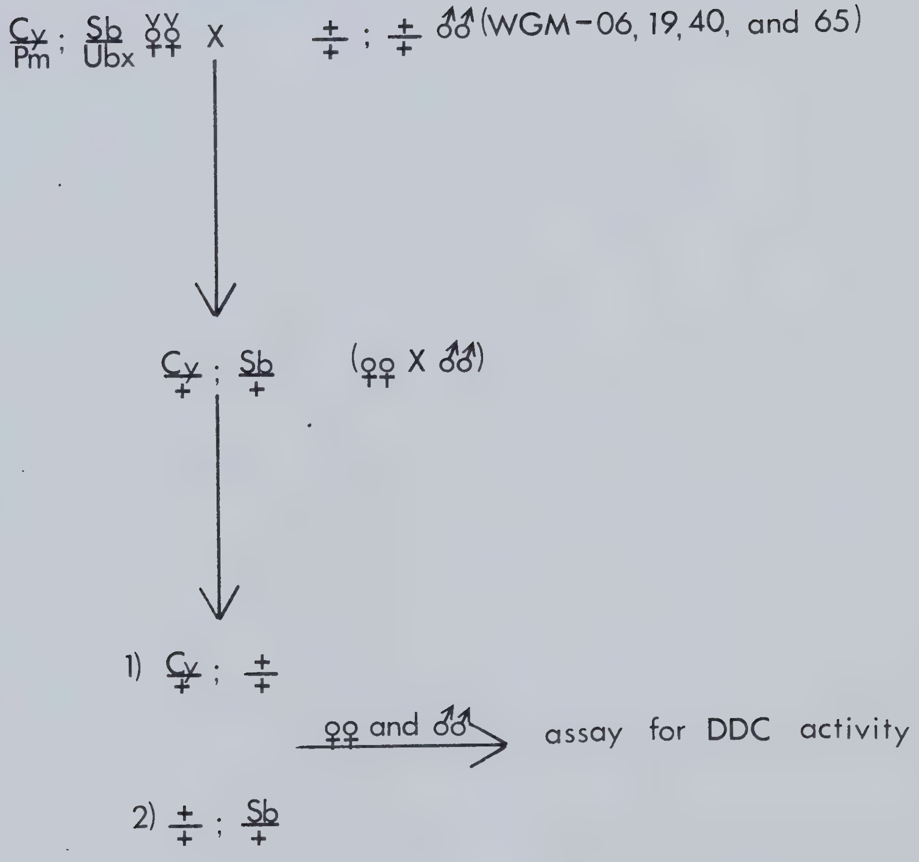
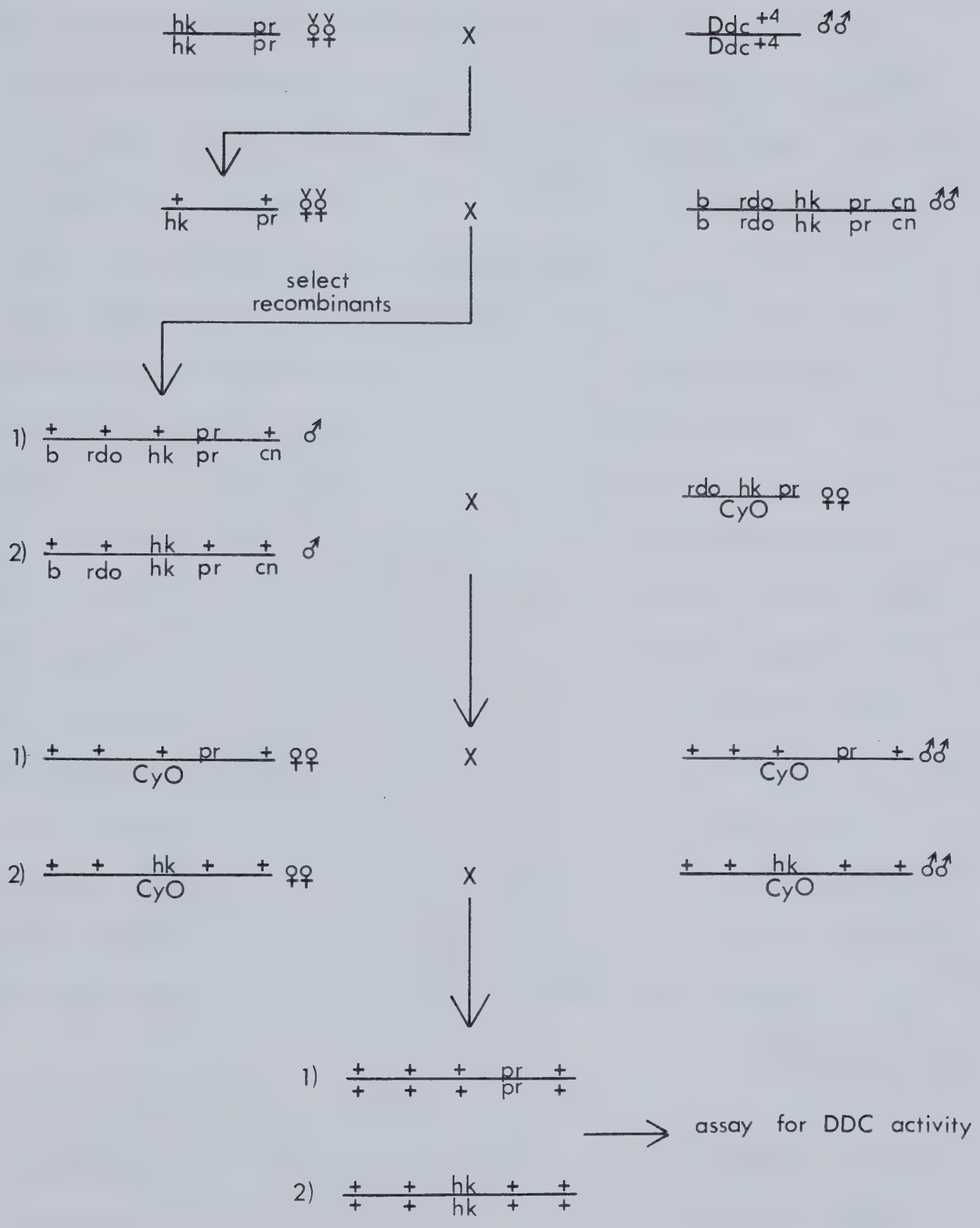

Cy/Pm ; Sb/Ubx ♀♀ X
+/+ ; +/+ ♂♂ (WGM-06, 19, 40, and 65)
Cy/+ ; Sb/+
(♀♀ X ♂♂)
1) Cy/+ ; +/+
♀♀ and ♂♂
assay for DDC activity
2) +/+ ; Sb/+

Figure 2: Crossing scheme used to test for sources of DDC activity variation between *hk* and *pr* on the left arm of chromosome 2. DDC assays were performed as described in Clark et al. (1978). Extracts of newly eclosed adults were prepared from equal numbers of male and female flies. White puparia were not sexed prior to being assayed for DDC activity.


hk pr / hk pr ♀♀
X
Ddc+4 / Ddc+4 ♂♂
+ + / hk pr ♀♀
X
b rdo hk pr cn / b rdo hk pr cn ♂♂
select recombinants
1) + + + pr + / b rdo hk pr cn ♂
2) + + hk + + / b rdo hk pr cn ♂
X
rdo hk pr / CyO ♀♀
1) + + + pr + / CyO ♀♀
X
+ + + pr + / CyO ♂♂
2) + + hk + + / CyO ♀♀
X
+ + hk + + / CyO ♂♂
1) + + + pr + / + + + pr +
2) + + hk + + / + + hk + +
assay for DDC activity

(1978) except that the ammonium sulfate precipitation step was not done. *Staphylococcus aureus* cells were prepared and supplied by Dan Gietz as in Kessler (1976).

Typically extracts were made at a concentration of 50 mg/ml of extraction buffer. The extract was divided into 25 μl aliquots and made up to 50 μl with antiserum solution diluted to the desired concentration in extraction buffer. All the samples were incubated for two hours at 4° C. To ensure that immunoprecipitation did not depend on the antibody to antigen ratio 10 μl of a 10% *S. aureus* suspension was added to act as immunoadsorbent. Incubation at 4° C for 30 minutes was followed by centrifugation at 8500 x g for 10 minutes. The supernatants were removed and centrifuged again at 8500 x g for 10 minutes. These supernatants were removed and frozen in liquid nitrogen. Each sample was assayed in duplicate and the fraction of the activity remaining calculated using the tube with no antiserum added as a reference. These numbers were plotted against serum concentration and that concentration yielding 50% enzyme inactivation determined from the graph.

G. *In vivo* radiolabelling of DDC

Approximately 50 mg of wandering third instar larvae were fed 100 μCi of ^{35}S methionine in 2% ethanol diluted with 65 μl of water in a small petri dish (Pass 1979). After this was completely ingested (approximately 4 hours) the larvae were fed a 1% sucrose solution for a period of 8 to

10 hours. Crude extracts were prepared in extraction buffer at a concentration of 50 mg/ml.

H. Immunoprecipitation of *in vivo* radiolabelled DDC

Preimmune IgG and anti-DDC IgG were generously supplied by Dan Gietz after purification on protein-A sepharose columns. Enzyme was immunoprecipitated as described in Kraminsky et al., (1979). A 300 µl volume of radiolabelled crude extract was incubated with 300 µl of 150mM NaCl, 5mM EDTA, 50mM Tris HCl (pH 7.2), 0.02% w/v sodium azide (NET) + .5% Triton X-100 and approximately 50 µg of preimmune IgG for 12 hours at 4° C. The IgG was precipitated by adding 100 µl of a 10% *S. aureus* suspension, incubating for 30 minutes at 4° C, and centrifuging at 2000 x g for 5 minutes. This was repeated with a further 100 µl of *S. aureus* suspension. Approximately 25 µg of anti-DDC IgG was added and the solution incubated at 4° C for 3 hours. The IgG-enzyme complexes were precipitated with 100 µl of *S. aureus* suspension as described above. Cells collected by centrifugation were washed 5 times with NET + .05% Triton X-100 and stored at -20° C.

I. Determination of alpha-methyl Dopa sensitivity

Standard yeast-sucrose media was supplemented with varying concentrations of α-methyl Dopa (αMD) made up fresh in .001 N HCl(Sherald and Wright 1974). The concentration of αMD usually ranged from $.5 \times 10^{-4}$ to 4×10^{-4} M with

nonsupplemented standard food as a control. Fifty newly hatched first instar larvae were set on squares of moistened filter paper. These filter papers were laid on top of 5 mls of food poured into small tubes. Viability was scored after all surviving larvae had pupariated and the % survival determined by reference to the control. Three tubes at each concentration were set up in each experiment. The mean number of survivors calculated using all three tubes was used to determine % survival at each concentration.

J. RNA extraction from *D. melanogaster*

All glassware used in RNA extractions was heat treated at 150° C for at least 4 hours to inactivate RNase. Extractions were performed from first instar larvae, wandering third instar larvae, and newly eclosed adults in the following extraction buffer: 250mM sucrose, 250mM NaCl, 250mM NH_4Cl, 50mM $MgCl_2$, 25mM Tris HCl (pH 7.5), 5mM EGTA, 5mM N-ethylmalemide, 100 μg/ml heparin, .5% v/v 2-mercaptoethanol, 2% w/v SDS, 50 μg/ml spermine (Benyjati et al.1981). This buffer was made up in double distilled water that had been made .05% in diethyloxidiformate and autoclaved. Third instar larvae and adults were ground to a fine powder in liquid nitrogen using a precooled mortar and pestle. This powder was placed in a glass grinder and homogenized at a concentration of 20 ml extraction buffer to 1 gm of material. First instar larvae were treated the same way except that grinding in liquid nitrogen was not

required. After grinding, the homogenate was made 1% v/v NP 40 and vortexed for several seconds. A .5 volume of phenol saturated with 50mM Tris HCl (pH 7.5) was added and the solution again vortexed for several seconds. Next a .5 volume of chloroform was added and the solution hand agitated for several minutes. Centrifugation at 12,000 x g for 10 minutes separated the phases. The aqueous phase was removed and extracted 2x with equal volumes of chloroform. Finally the RNA was precipitated by making the solution 200mM sodium acetate (pH 7.0), adding 3 volumes of 95% ethanol, and placing at -20° C for at least 8 hours.

K. DNA extraction from *D. melanogaster*

This procedure for the isolation of high molecular weight DNA was adapted from that of Schachat and Hogness (1974). Typically DNA was extracted from embryos 0-24 hours old. However this procedure was applied to larvae and adults as well.

Embryos were dechorionated by bathing in 2.5% sodium hypochlorite for 90 seconds and homogenized at a ratio of 1:10 w/v in 150mM NaCl, 50mM EDTA, 1% w/v sarkosyl (pH 7.8) (NES) in a Bellco glass homogenizer. The debris was removed by centrifugation at 12,000 x g for 10 minutes. The supernatant was mixed with one volume of phenol saturated with 10mM Tris HCl (pH 7.8), 1mM EDTA. Extraction proceeded for 1 hour with gentle shaking at 4° C. The phases were separated by centrifugation at 12,000 x g for 15 minutes.

After removing the aqueous phase, the phenol phase was re-extracted with .5 volume of NES for 30 minutes with gentle shaking. The aqueous phase was removed after centrifugation and pooled with the first aqueous phase. This solution was again extracted with phenol at room temperature. After centrifugation the aqueous phase was extracted 4x with ether and the DNA precipitated with 3 volumes of 95% ethanol at -20° C overnight. The DNA was collected by centrifugation at 4,000 x g in a GSA rotor for 20 minutes, dried under vacuum, and resuspended in 7.6 ml TE. Cesium chloride (7.38 gm) and ethidium bromide (400 µl of a 10 mg/ml solution) were added and the solution placed in 2 SW 50.1 nitrocellulose centrifuge tubes. The DNA was banded by centrifugation at 36,000 rpm at 15° C for 48 hours. The DNA was recovered with a syringe and the ethidium bromide removed by extracting 3x with water saturated n-butanol. Finally the DNA was dialyzed against 1,000 ml of TE with 3 changes at 4° C.

L. Oligo dT chromatograhy

Oligo dT chromatography was performed according to Aviv and Leder (1972). Approximately .25 gms. of oligo dT-cellulose was equilibrated in 10mM Tris HCl (pH 7.4), 400mM NaCl, .5% w/v SDS and placed in a pasteur pipette. Bulk RNA collected by centrifugation and resuspended in equilibration buffer was applied to the column at a rate of 12 mls/hour. After sample application the column was washed

with equilibration buffer. The absorbance at 260 nm was monitored continuously with an ISCO UV analyzer and flow cell. When the absorbance had returned to baseline the poly A^+ RNA was released from the column with 10mM Tris HCl (pH 7.5), .05% w/v SDS. The poly A^+ RNA sample was rechromatographed until no RNA was detected in the wash. Frequently this required 3 passes over the column. Finally the RNA sample was made 200mM in sodium acetate (pH 7.0) and precipitated in 3 volumes of ethanol at -20° C for 8 hours. The precipitate was resuspended in a small volume of distilled water and stored at -70° C.

M. Gel electrophoresis

SDS polyacrylamide gel electrophoresis

In vivo radiolabelled DDC was analyzed on SDS polyacrylamide vertical slab gels according to Kikuchi and King (1975). Samples were prepared by diluting 1:1 in cracking buffer (3% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol, 62mM Tris HCl (pH6.8)) and boiling for 5 minutes. *S. aureus* cellular debris was removed by centrifugation at 2000 x g for 5 minutes. A 10% separating gel and a 4.5% stacking gel were used, both 30:1 acrylamide to bis. Electrophoresis was carried out at 90 volts for 3 to 4 hours at room temperature. Proteins were fixed in the gel by bathing in 10% v/v acetic acid solution for at least 3 hours. To remove the acid in preparation for fluorography

the gel was incubated in 20 volumes of water for 30 minutes (Chamberlain, 1979). It was then incubated in 10 volumes of 1M sodium salicylate (pH 7.0) for approximately 1 hour. The gel was dried under vacuum and Kodak XRP-1 x-ray film was exposed to the dried gel at -70° C.

DNA polyacrylamide gel electrophoresis

DNA restriction fragments were analyzed on 3.5% polyacrylamide (30:1 acrylamide bis) vertical slab gels (Maniatis et al.,1982). Gels were cast and run in 90mM Tris-borate (pH 8.0), 2mM EDTA. Electrophoresis was for 3 to 4 hours at 80 volts. Samples were loaded in .03% xylene cyanol, .03% bromophenol blue, 10% glycerol.

Denaturing RNA agarose gel electrophoresis

Agarose electrophoresis of RNA was performed essentially as in Derman et al., (1981). RNA samples were denatured by heating at 65° C for 5 minutes in 50% v/v formamide, 6% v/v formaldehyde, 20mM sodium borate (pH 8.3), .2mM EDTA. After denaturation the samples were made .03% xylene cyanol, .03% bromophenol blue, 10% glycerol. The samples were loaded onto a horizontal 1.25% gel made in 20mM sodium borate (pH 8.3), .2mM EDTA, 3% v/v formaldehyde and run at a constant current of 100mA for 3 to 6 hours. The running buffer was recirculated to prevent the formation of a pH gradient from anode to cathode. Yeast ribosomal RNAs were used as molecular weight markers. Marker RNA was

visualized using long wavelength UV light after staining in .5 μg/ml ethidium bromide for at least 4 hours.

DNA agarose electrophoresis

DNA preparations were routinely analyzed on horizontal submerged agarose gels ranging in agarose concentration from .4 to 1.5 %. Gels were cast and run in 40mM Tris-acetate (pH 8.0), 2mM EDTA (TAE) at approximately 30 volts for 12 to 24 hours at room temperature. The running buffer contained ethidium bromide at a concentration of .5 μgs/ml Samples were loaded as described for DNA polyacrylamide gels. After electrophoresis the DNA was visualized with long wavelength UV light and photographed with a Polaroid 545 Land camera.

Cellulose acetate electrophoresis

Extracts were prepared in standard extraction buffer and run on gelatinized cellulose acetate (cellogel, Chemetron) strips in a 50mM barbitol (pH 8.8) buffer at 200 volts for 30 minutes. Dopa decarboxylase was visualized by an antibody overlay technique using DDC specific antiserum. First crude serum, diluted in 10mM sodium phosphate (pH 7.3), 150mM NaCl, was applied to the gel by saturating a cellulose acetate membrane in antiserum solution and laying this membrane over the cellulose acetate gel. This was incubated at 4° C for 2 hours. Then the overlay membrane was replaced with another membrane, this time saturated with ^{125}I labelled anti-DDC IgG. This

antibody was purified by protein A-sepharose chromatography and iodinated using the lacto-peroxidase method (Bio-Rad) by the Edmonton Radiopharmaceutical Center. After an incubation period of 20 hours the gels were washed with 1M NaCl, 10mM sodium phosphate (pH 7.3), 1% Triton X-100 for 24 hours with 2 buffer changes. The gels were then air dried and exposed to x-ray film.

Agarose gel purification of DNA restriction fragments

Restriction fragments were purified using low melting temperature agarose (Sigma) according to Maniatis et. al. (1982). A 1% low melting temperature agarose gel was cast in TAE at 4° C in a horizontal slab gel apparatus. The DNA sample was loaded as described above and run in TAE buffer with ethidium bromide at 30 volts and at 4° C until such time as the restriction fragment of interest was well separated from other DNA fragments. The DNA band was cut out of the gel, placed in 5 volumes of TE, and incubated at 68° C for 10 minutes. The solution was extracted with 1 volume of TE saturated phenol at room temperature and the phases separated by centrifugation at 12,000 x g for 10 minutes at room temperature. The phenol phase was re-extracted with a .5 volume of TE, again at room temperature. The pooled aqueous phases were extracted with phenol-chloroform (1:1) and then chloroform. If necessary the DNA was concentrated with secondary butanol before precipitation in 3 volumes of 95% ethanol at -70° C for 1 hour.

N. Northern analysis

The method used for transfer of RNA is essentially as described by Southern (1975) for application to DNA transfer with some modifications (Derman et. al. 1981). Gels were not stained after electrophoresis but instead placed directly on a wick composed of 12 layers of Whatman 3mm paper saturated with 10 x SSC buffer. The gel was overlaid with a piece of nitrocellulose filter (Schleicher and Schuell, BA85) that had been equilibrated first with distilled water and then with 10 x SSC. This was then overlaid with 5 pieces of Whatman 3MM paper saturated with distilled water and about 5 cm of dry paper towel weighted down with a full 1 liter flask. Blotting was allowed to proceed for 12 to 18 hours. At completion the nitrocellulose filter was removed and baked for 2 hours at 80° C under vacuum.

O. Southern analysis

Southerns were done as described by Southern (1975) with some modifications as outlined in Davis et al., (1980). DNA samples were run on 1% agarose gels using TAE as the buffer system. Before blotting the gels were bathed in .25N HCl at room temperature for 30 minutes with one solution change. This was followed by 30 minutes at room temperature in .5M NaOH, 1.5M NaCl with one change and 30 minutes in .5M Tris HCl (pH7.5), 1.5M NaCl again at room temperature. All subsequent steps were done as described for northern analysis except that 20 x SSC was used in place of 10 x SSC.

P. Hybridization of radiolabelled DNA probes to nucleic acid blots

Hybridization of nick-translated DNA probes to blots was done according to Thomas (1980). Each filter was prehybridized in a 10 ml volume of 50% v/v formamide, 5 x SSC, 50mM sodium phosphate (pH 6.5), 250 μg/ml sonicated and denatured salmon sperm DNA, and .02% each of bovine serum albumin, ficoll and polyvinylpyrolidine for 2 to 8 hours at 42° C. Hybridization buffer consisted of 4 parts prehybridization solution and 1 part 50% w/v dextran sulfate. DNA probes were denatured by heating to 95° C for 5 to 10 minutes and added to 10 ml of hybridization mix. Hybridization was for 12 to 20 hours at 42° C with shaking. The blots were washed with 4 changes of 2 x SSC, .1% SDS for 5 minutes each at room temperature followed by two 15 minute washes with .1 x SSC, .1% SDS at 50° C. Blots were wrapped in saran wrap and Kodak XRP-1 x-ray film was exposed to the blots using an intensifying screen at -70° C.

Q. Estimation of relative Ddc transcript levels

Autoradiographs of northern blots were scanned using a Perkin Elmer 559 A spectrophotometer with a gel scanning attachment. The intensity of a band was measured by cutting out and weighing the peak produced by the recording unit of the spectrophotometer. This value in milligrams was then corrected for the amount of RNA loaded on the gel. Each gel typically consisted of 4 slots of each sample to be

analyzed. The 4 intensity values for each RNA type were then averaged to obtain a mean. In order to ensure that the values produced were within the linear range of the system a standard curve of increasing amounts of RNA loaded was included on each gel.

R. Enzymatic treatment of DNA

Endonuclease restriction

All restriction endonucleases were used as recommended by the manufacturer. Typically 1 μg of DNA was restricted in a volume of 15 μl with 5 units of enzyme at a temperature of 37° C for 1 hour. Reactions were stopped by incubation at 68° C for 10 minutes. The volume of the reaction and the time of incubation were increased for those DNA samples found to be slow to digest.

Ligation of DNA with T4 ligase

The DNA samples to be ligated were precipitated together in 3 volume of 95% ethanol, washed 1 x with 70% ethanol and resuspended in 50mM Tris HCl (pH 7.8), 10mM $MgCl_2$, 2mM dithiothreitol, 1mM adenosine triphosphate, 2mM spermidine, 50 μg/ml bovine serum albumin. After addition of .1 unit of T4 DNA ligase the reaction was incubated at 14° C for 2 to 12 hours.

Nick-translation of DNA

Radiolabelled nucleotides were incorporated into DNA by nick-translation as in Davis et al., (1980). Typically, reactions took place in a 25 μl volume. The reaction mixture contained 50mM Tris HCl (pH 7.5), 100mM $MgSO_4$, 1mM dithiothreitol, 50 μg/ml bovine serum albumin, .02mM dATP, dGTP, and dTTP, approximately 50 μCi ^{32}P dCTP, 5 units of DNA polymerase 1, .125 ng DNase 1 and .5 μg DNA. Incubation was at 14° C for 3 hours and the reaction was stopped by addition of an equal volume of 200mM EDTA, 2mg/ml sonicated salmon sperm DNA, .2% SDS. Unincorporated nucleotides were removed by passing the reaction over a Sephadex G-50 column in a pasteur pipette equilibrated with TE. The first .5 to 1 ml containing radioactivity as monitored by a Mini Instruments Geiger meter was collected. Total incorporation was measured by spotting a 1 μl aliquot of nick translated DNA onto a Whatman DE-81 filter. The filter was placed in a Millipore filtration apparatus and washed with 30 ml of 300mM ammonium formate (pH 7.8). After allowing the filter to air dry it was placed in 10 mls of Aquasol-2 scintillation fluid (New England Nuclear) and counted in a Beckman scintillation counter.

S. Growth and handling of bacteriophage lambda

Most of these procedures are adapted from Maniatis et al. (1982) and Davis et al., (1980).

Plating of lambda phage

Plating culture was prepared by harvesting a freshly saturated culture of λ sensitive cells and resuspending the pellet in .4 volumes of TMG buffer. This culture was stored at 4° C and used for approximately 1 week. Phage suspension, diluted to give 50 to 500 plaques per plate, was added to .1 ml of plating culture and allowed to preadsorb for 20 minutes at 37° C. Then 3 ml of LMB soft agar heated to 45° C was added and the mixture poured onto a prewarmed LB plate. After the agar had hardened the plates were incubated upside down at 37° C for 12 to 15 hours.

Preparation of plate lysate

Approximately 10^6 phage were plated per plate as described. The plates were incubated right side up for approximately 12 hours or until lysis was almost confluent. Then 5 mls of TMG was added to each plate and the soft agar scraped into a centrifuge tube with a glass rod. A few drops of chloroform were added and the mixture vortexed violently for 30 seconds. The lysates were incubated at room temperature for 30 minutes and then centrifuged at 10,000 x g for 15 minutes. The clarified lysates were stored at 4° C with a few drops of chloroform at the bottom of the tube.

Preparation of liquid lysate

Typically a 250 ml volume of LMB was inoculated with a 3 ml fresh overnight of λ sensitive cells and incubated at 37° C with shaking until an OD^{650} of .4 was attained. Then the culture was infected with phage at a multiplicity of infection of approximately 5. Preadsorption took place for 20 minutes at 37° C. This was followed by from 3 to 4 hours of vigorous shaking at 37° C. A volume of 2.5 ml of chloroform was added to each culture and shaking at 37° C continued for a further 20 minutes. DNase and RNase were added to a concentration of 1 μg/ml and the culture allowed to sit for 1 hour at room temperature. Then NaCl was added to a concentration of 1M and the solution cleared by centrifugation at 10,000 x g for 10 minutes. The lysate was stored at 4° C.

Preparation of lysate from a single plaque

A lysate was prepared from a single plaque by removing the plaque and soft agar with a sterile toothpick or pasteur pipette and placing it in .5 ml of TMG. A few drops of chloroform were added and the solution vortexed vigorously for 30 seconds. The solution was allowed to sit for at least 1 hour at room temperature or overnight at 4° C before plating. A lysate prepared in this way typically contained approximately 10^5 phage particles.

Purification of bacteriophage lambda

A 250 ml liquid lysate prepared as described above was made up to 10% w/v with polyethylene glycol 6000 (PEG) and incubated for 1 hour at 4° C. The PEG precipitate was collected by centrifugation at 10,000 x g for 20 minutes, drained completely and resuspended in 2 mls of TMG. The PEG was removed by 2 chloroform extractions. The phage were then further purified by 2 CsCl block gradients as described in Davis et al., (1980).

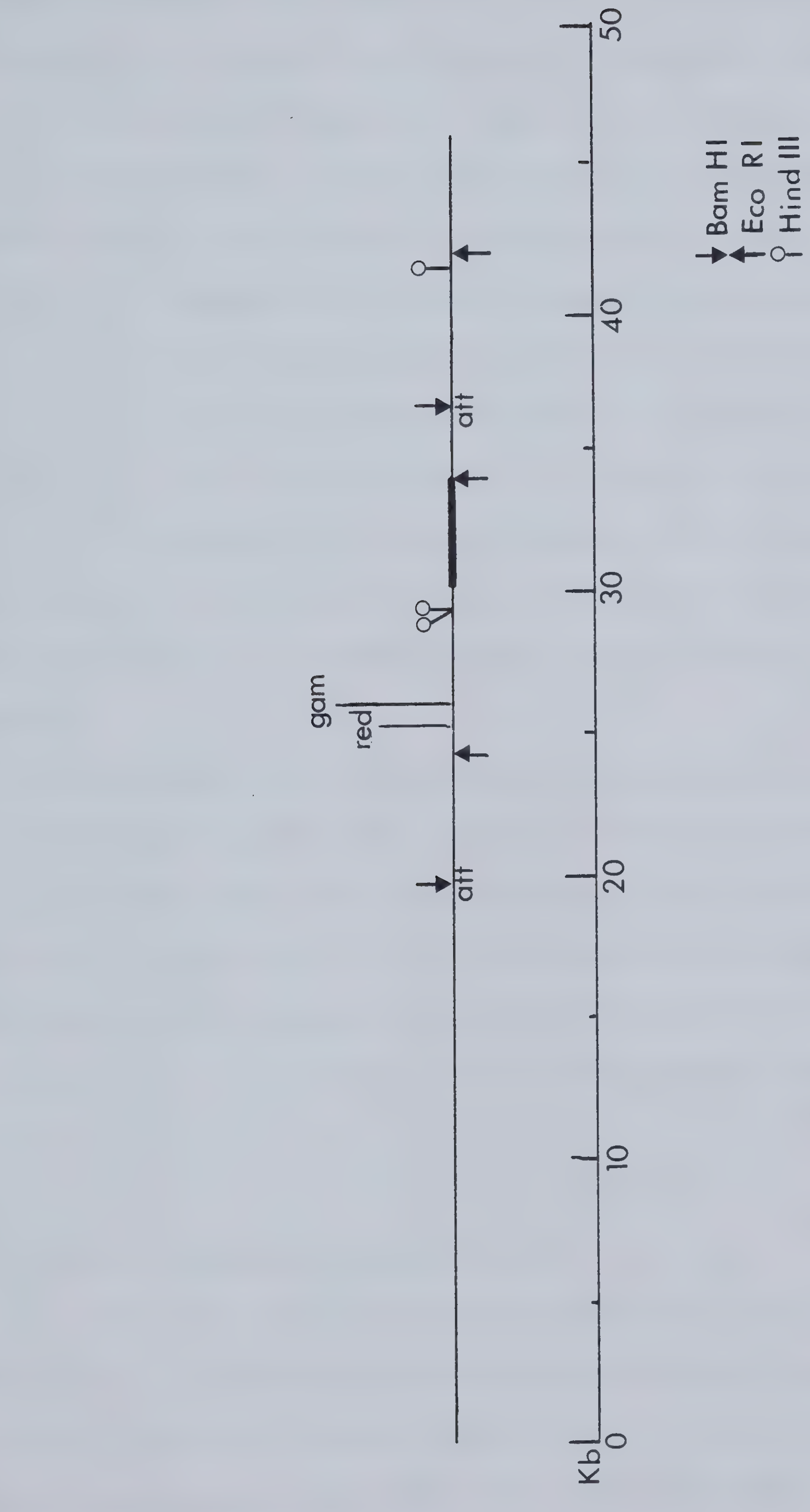
Extraction of DNA from bacteriophage lambda

After the phage band was removed from the second CsCl block gradient the solution was made 200mM Tris HCl (pH7.5), 20mM EDTA. One volume of formamide was added and the solution incubated at least 1 hour at room temperature. Then 1 volume of water and 6 volumes of 95% ethanol were added. The phage DNA precipitated immediately and was pelleted by spinning in an Eppendorf microfuge for 5 minutes. The DNA was washed once with 70% ethanol, resuspended in a small volume of TE, and stored at 4° C.

T. Construction of a *D. melanogaster* library in lambda 1059

The bacteriophage λ 1059 (Karn et al., 1980) was chosen as the vector to be used in construction of a recombinant DNA library. A restriction map of λ1059 DNA is presented in Figure 3. This phage has 2 characteristic advantages. Firstly, because it is a Bam H1 substitution vector it is

Figure 3: Restriction map of bacteriophage λ1059. The location of restriction sites in λ1059 was obtained from Karn et al. (1980). The bold line indicates pBR322 sequence.


Kb
0
10
20
30
40
50
att
red
gam
att
Bam HI
Eco RI
Hind III

possible to insert Sau 3A restriction fragments since these 2 enzymes generate an identical single stranded terminus. Sau 3A has a 4 nucleotide recognition sequence. Consequently it cuts relatively frequently and a partial digestion will yield an essentially random collection of fragments. Secondly use of the *spi* phenotype allows for positive selection of recombinant phage. The *spi*$^+$ phenotype is conferred by the *red* and *gam* genes both of which are situated on the central "stuffer" Bam H1 fragment of λ1059 (Figure 3). A *spi*$^+$ bacteriophage λ is unable to replicate in a host cell lysogenic for bacteriophage P2. A recombinant phage replacing the central Bam H1 fragment with *Drosophila* DNA would have a *spi*$^-$ phenoype and would grow on a P2 lysogen.

The strategy for construction of a recombinant library with λ1059 was to partially restrict *Drosophila* DNA with Sau 3A, fractionate the resulting fragments on a sucrose gradient, ligate the 15 to 20 Kb fragments into Bam H1 restricted λ1059, package the product phage DNA *in vitro*, and select for recombinants on a host lysogenic for phage P2.

Sau 3A restriction of *Drosophila* DNA

In order to find the correct conditions for partial restriction, .5 μg aliquots of *Drosophila* DNA were digested with from .01 to 1.0 units of Sau 3A in 6mM Tris HCl (pH7.5), 6mM $MgCl_2$, 50mM NaCl for 1 hour at 37° C. Each

sample was run on a .8% agarose gel in TAE with molecular weight size markers (Figure 4A). Those conditions which produced the most DNA in the 15 to 20 Kb range were chosen and the reaction scaled up exactly for digestion of 150 µg of DNA. After restriction for 1 hour at 37° C the DNA was extracted once with phenol chloroform (1:1), twice with chloroform, made up to 200mM sodium acetate, precipitated in 3 volumes of 95% ethanol at -70° C for 1 hour, collected by centrifugation, and resuspended in 500 µl of TE buffer.

Sucrose gradient fractionation of restricted DNA

The 500 µl aliquot of restricted DNA was incubated at 68° C for 10 minutes, cooled to room temperature and loaded on top of a 10% to 40% w/v sucrose gradient in 50mM Tris HCl (pH 7.8), 100mM NaCl, 10mM EDTA in a SW 27 polyallomer centrifuge tube. This gradient was centrifuged in a SW 27 rotor for 24 hours at 25,000 rpm and 15° C. The gradient was fractionated into 500 µl fractions on an ISCO gradient fractionator. A 10 µl aliquot from every second fraction was run on a .5% agarose gel in TAE with molecular weight markers (Figure 4B). Those fractions containing DNA of primarily 15 to 20 Kb were pooled, dialyzed into 3 changes of 1000 ml of TE, and stored at 4° C.

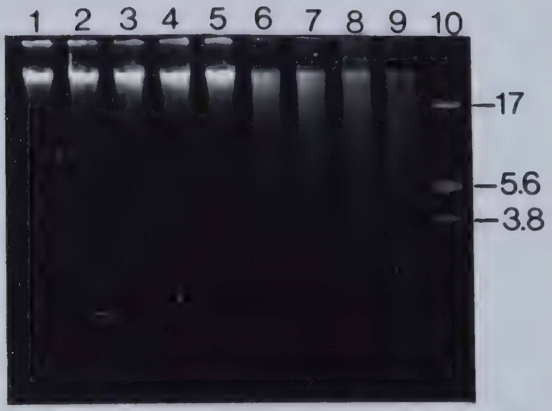
Preparation of *in vitro* packaging extracts

The procedure for preparation of packaging extracts is essentially the same as that described in Maniatis et

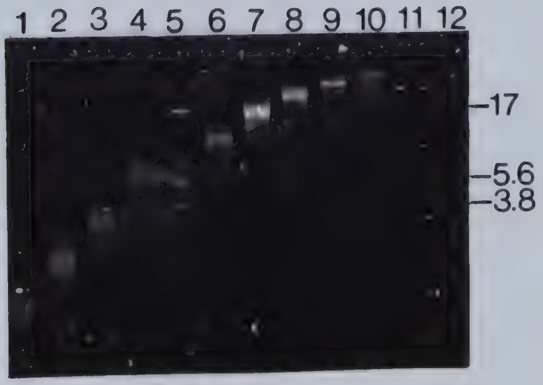
Figure 4A: Partial restriction of *Ddc*$^{+4}$ DNA. Aliquots of approximately 0.5 μg of high molecular weight DNA were restricted with from 0.01 to 1.0 units of Sau 3A restriction enzyme for 1 hour at 37°C, and electrophoresed on 0.8% agarose gel (slots 1 through 9). Slot 10 contains restricted DNA from a recombinant Charon 4A bacteriophage. These fragments served as molecular weight markers (Kb). Flourescence in the 17 Kb size range is the most intense in slot 6. The conditions used for restriction of the DNA in slot 6 were therefore used for large scale restriction.

Figure 4B: Size fractionation of *Drosophila* DNA. Approximately 150 μg of *Drosophila* DNA was restricted with Sau 3A according to conditions determined above. This DNA was centrifuged on a sucrose gradient as described in the text. The gradient was fractionated and aliquots from fractions throughout the gradient were electrophoresed on 0.4% agarose gel (slots 1 to 4 and 6 to 12). Slot 5 contains marker DNA as described above. The DNA in slot 7 is of the desired size and was used for library construction.

A


1 2 3 4 5 6 7 8 9 10
17
5.6
3.8

B


1 2 3 4 5 6 7 8 9 10 11 12
17
5.6
3.8

al. (1982).

Sonicated extracts

A 500 ml volume of LMB was innoculated with 3 ml of a fresh overnight of strain BHB 2690 grown at 30° C. This culture was incubated at 30° C until an OD^{650} of .3 was reached and then incubated in a 45° C water bath for 20 minutes to induce production of λ proteins. Following induction the culture was incubated at 37° C with vigorous shaking for 3 hours. Cells were harvested as described and resuspended in 3.6 mls of 20mM Tris HCl (pH8.0), 1mM EDTA, 3mM $MgCl_2$, 5mM 2-mercaptoethanol (sonication buffer). The solution was sonicated with 10 second bursts on high setting at 4° C until the solution cleared and the viscosity decreased. Debris was removed by centrifugation at 5,000 x g for 10 minutes. An equal volume of cold sonication buffer was added to the supernatant as well as 1/6 volume of 6mM Tris HCl (pH 8.0), 50mM spermidine, 50mM putrescine, 20mM $MgCl_2$, 30mM ATP, 30mM 2-mercaptoethanol (packaging buffer). Aliquots of 15 μl were frozen in liquid nitrogen and stored at -70° C.

Freeze-thaw extracts

A 500 ml induced culture of BHB 2688 was prepared exactly as described above for BHB 2690. The cells were resuspended after harvesting in 1 ml of cold 10% w/v sucrose, 50mM Tris HCl (pH 7.5). This solution was

placed in two 1.5 ml Eppendorf tubes, mixed with 25 μl of 2 mg/ml fresh lysozyme in 250mM Tris HCl (pH 7.5), and frozen in liquid nitrogen. The frozen mixtures were thawed on ice. A 25 μl volume of packaging buffer was added to each tube and the suspension was centrifuged at 48,000 x g for 1 hour at 4°C. The supernatant was divided into 10 μl aliquots, frozen in liquid nitrogen, and stored at -70° C.

In vitro packaging

Tubes containing aliquots of freeze thaw and sonicated packaging extracts were removed from the -70° C freezer, allowed to thaw on ice, and mixed. Then 2 μl aliquots of ligation mixture were mixed with the packaging extracts and incubated at room temperature for 1 hour. A volume or 250 μl of TMG phage buffer was then added to each tube. All of the aliquots were pooled in preparation for plating.

Amplification of the library

The library obtained after packaging was amplified by plating onto strain Q358 at high density to yield a high titer plate lysate.

U. Library screen

Filter preparation

The λ:Drosophila library was screened essentially as described in Maniatis et al., (1982). Approximately 10,000 plaques were plated in .3 mls of Q358 plating culture. In place of agar, .7% agarose was used in the top layer. Plaques were allowed to develop for approximately 12 hour and then the plates were cooled to 4° C for at least 1 hour. Dry nitrocellulose filters (Schleicher and Schuell) were placed carefully on the cooled plates and allowed to adsorb phage DNA for 1 minute. India ink applied with a syringe was used to mark the filter and plate simultaneously for later orientation. The filter was removed from the plate and immersed sequentially in; 1.5M NaCl, .5M NaOH for 1 minute, 1.5M NaCl, .5M Tris HCl (pH 8.0) for 2 x 5 minutes, and 6 x SSC for 2 x 5 minutes. A duplicate filter was prepared from each plate. This second filter was allowed to sit on the plate for 2 minutes. After the filters had air dried they were baked in a vacuum at 80° C for 2 hours.

Hybridization

Prehybridization and hybridization were performed as described earlier except that hybridization was done without dextran sulfate.

V. Transformation of *Escherichia coli* cells with plasmid DNA

Transformation of *E. coli* cells was done according to Cohen et al., (1972). A fresh saturated culture of strain HB 101 was diluted 1/40 in LB and incubated at 37° C with shaking for 1 hour. Cells were harvested by centrifugation and resuspended in a .5 volume of cold 50 mM $CaCl_2$. Cells were immediately pelleted again and resuspended in a .1 volume of cold 50mM $CaCl_2$. This suspension was then incubated on ice for at least 1 hour before use.

For transformation a 10 to 20 μl volume of DNA was added to .1 ml of competent cells. After incubation on ice for 30 to 60 minutes the cells were heat shocked at 45° C for 2 minutes. A .3 ml volume of LB was added to each transformation and the culture incubated at 37° C for 45 to 60 minutes. Dilutions were plated onto LB plates with appropriate antibiotics. The plates were incubated for 12 to 24 hours at 37° C.

W. Extraction of plasmid DNA

Plasmid DNA was isolated as described in Maniatis et al. (1982). A 25 ml volume of LB + antibiotic was inoculated with .1 ml of a saturated culture and grown at 37° C with shaking until an OD^{650} of .6 was reached. This culture was then used to inoculate 500 ml of prewarmed LB + drug. The culture was shaken vigorously at 37° C for 2.5 hours. Then 2.5 ml of a 34 mg/ml chloramphenicol solution was added to the 500 ml culture. Vigorous shaking at 37° C was continued

for 14 to 16 hours. The cells were harvested and resuspended in 10 ml of 10% w/v sucrose in 50mM Tris HCl (pH 8.0). To this solution 2 ml of 10 mg/ml fresh lysozyme in .25M Tris HCl (pH 8.0) and 8 ml of .25M EDTA was added. The tube was then left on ice for 10 minutes. Chromosomal DNA was precipitated by addition in rapid succession and with gentle mixing, 4 ml of 10% w/v SDS followed by 6 ml of 5M NaCl. After incubation on ice for 1 hour the solution was centrifuged at 48,000 x g for 30 minutes. The supernatant was extracted twice with TE saturated phenol chloroform (1:1) and once with chloroform. Plasmid DNA was precipitated in 3 volume of 95% ethanol at -70° C for 1 hour, washed in 70% ethanol, harvested by centrifugation and resuspended in 8 ml of TE. For every ml of DNA solution 1 gm of CsCl was added. Ethidium bromide was added to a concentration of .8 μg/ml and the solution centrifuged at 45,000 rpm in a 50Ti rotor for 36 hours at 20° C. The plasmid band was removed by syringe and the ethidium bromide removed by extraction with water saturated n-butanol. Cesium chloride was removed by dialysis into 1,000 ml of TE with 3 changes and the DNA stored at 4° C.

X. Rapid extraction of plasmid DNA

Plasmid DNA was isolated for analytical purposes according to Birnboim and Doly (1979). A .5 ml volume of fresh saturated was centrifuged for 1 minute in an Eppendorf microfuge and the pellet resuspended in 100 μl of 2 mg/ml

fresh lysozyme, 50mM glucose, 10mM EDTA, 25mM Tris HCl (pH 8.0). After incubation on ice for 30 minutes 200 μl of .2M NaOH, 1% SDS was added and the solution vortexed until viscous. Chromosomal DNA and protein were precipitated by addition of 150 μl of 3M sodium acetate (pH 4.8) and incubation on ice for 60 minutes. The supernatant was cleared by centrifugation for 5 minutes in a microfuge. A 400 μl volume of supernatant was removed and plasmid DNA precipitated by addition of 1 ml of 95% ethanol and incubation at -70° C for thirty minutes. The DNA was resuspended in TE and reprecipitated in 95% ethanol, washed once with 70% ethanol and resuspended in a volume of 40 μl TE. A 5 μl volume of this DNA was usually sufficient for 1 restriction. Often RNase A was added to a concentration of .4 mg/ml during restriction.

III. Results

A. DDC activity and ecdysterone titer during post embryonic development

As a necessary prelude to the analysis of DDC activity variants, the level of DDC activity was determined in a Canton-S strain throughout post-embryonic development. In addition, the ecdysterone titer was determined during the 3 larval stages. These data complement embryonic and post-puparial data obtained in our laboratory and together constitute the profile plotted in Kraminsky et al., (1980) and presented here in Figure 5.

B. Survey of DDC activity

In an attempt to identify natural variation in DDC activity Dr. Ross Hodgetts and Bhagya Venkataraman surveyed enzyme activity at adult eclosion in 109 different strains. These strains were gifts of Dr. Glenn Bewley and Dr. George Carmody. They were originally constructed by isogenization of 109 different wild-type second chromosomes. The results of the survey are summarized in Figure 6. Enzyme activity for the 109 strains is expressed relative to Canton-S. The arithmetic mean of the distribution is 91% and the median value is 89%. Figure 6 shows that the values range from 65% to 150%. However, it should be noted that the two strains at the upper extreme of the distribution contribute disproportionately to this range. Strains WGM-06, WGM-19,

Figure 5: DDC activity and ecdysterone titer during the life cycle of the Canton-S strain at 25° C. DDC assays were performed as described in Clark et al. (1978). Enzyme activity is indicated by open triangles and ecdysterone titer by open circles. The determination of ecdysterone titerwas done by Dr. J.D. O'Connor. Embryonic samples were collected by R.D. Gietz. Some of the DDC assays on pupal and adult samples were performed by W.C. Clark. This Figure has been published in Kraminsky et al. (1980).

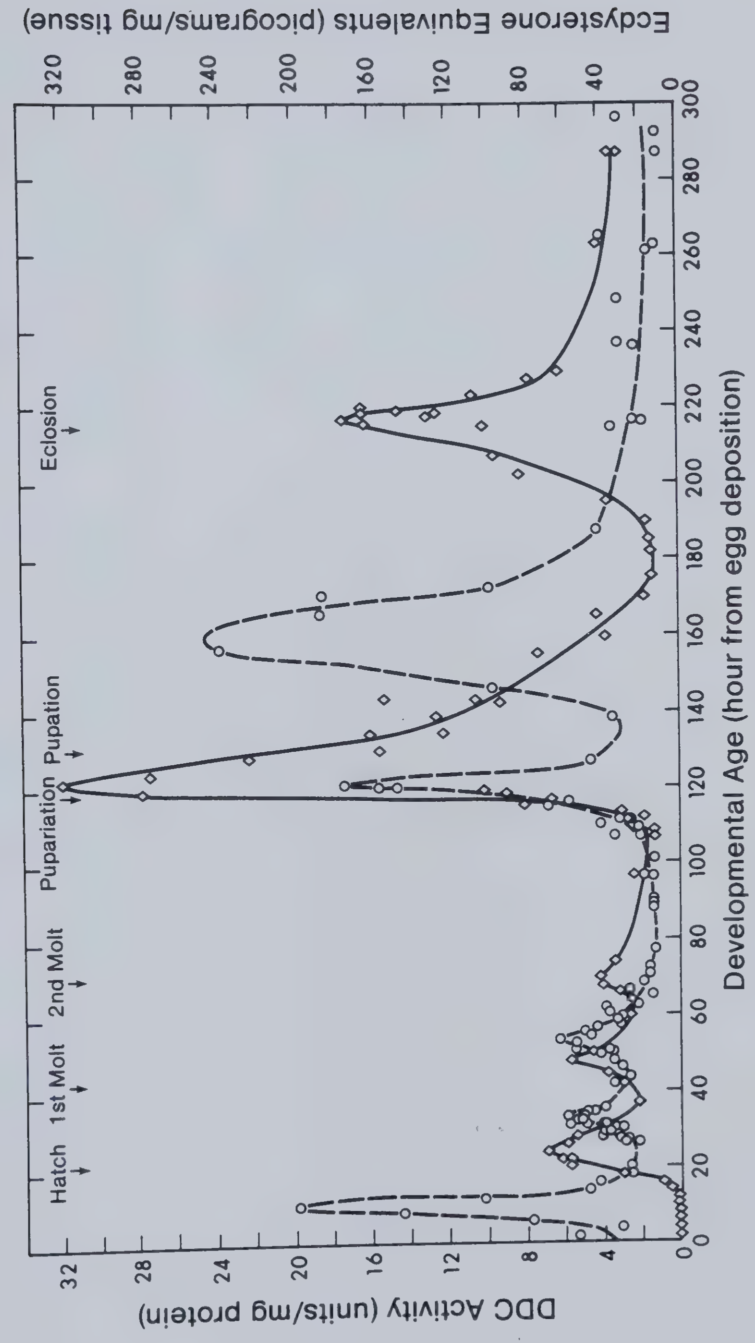
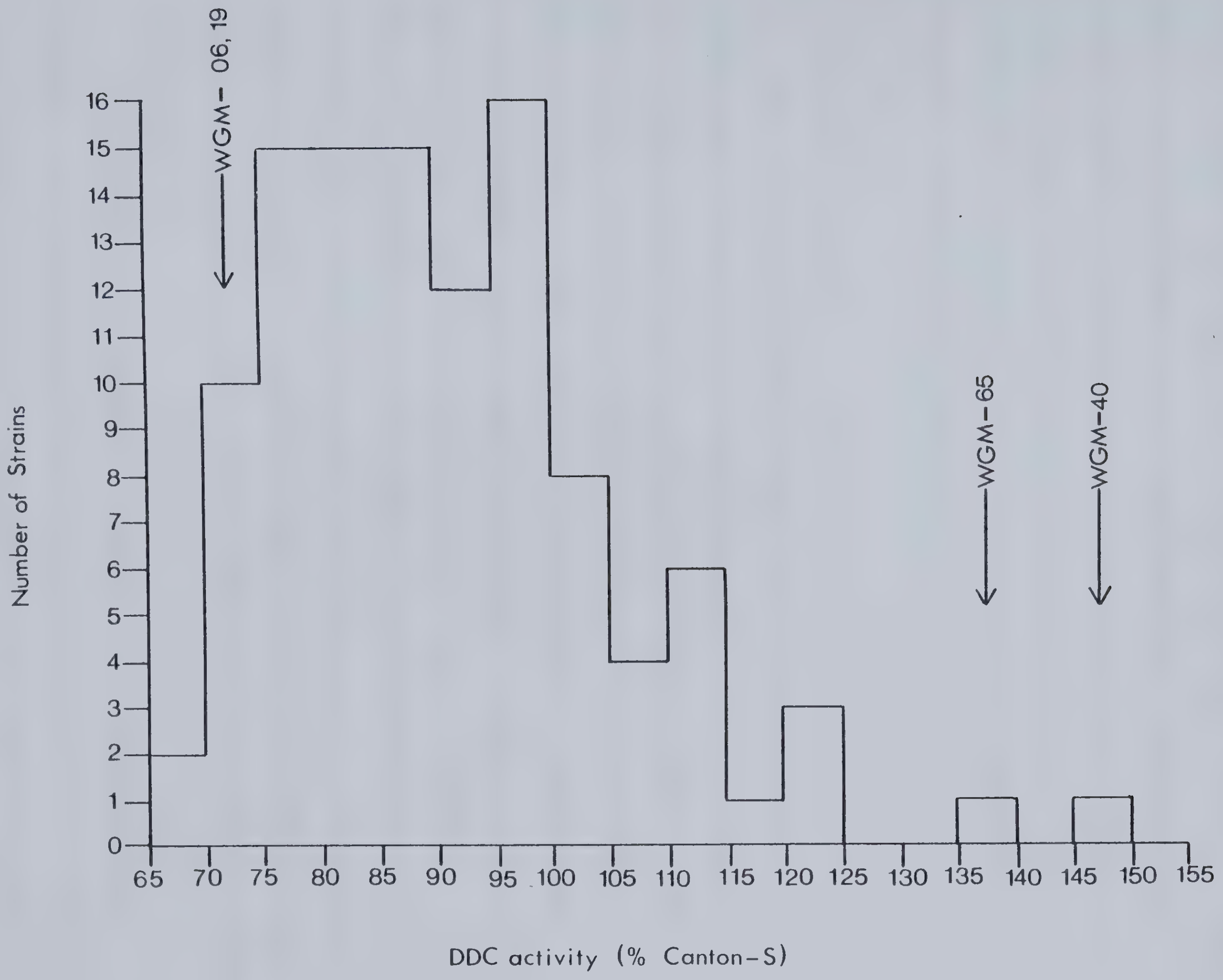

Hatch
1st Molt
2nd Molt
Pupariation
Pupation
Eclosion
DDC Activity (units/mg protein)
0
4
8
12
16
20
24
28
32
Ecdysterone Equivalents (picograms/mg tissue)
0
40
80
120
160
200
240
280
320
Developmental Age (hour from egg deposition)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Figure 6: Survey of DDC activity in strains isogenic for independently isolated wild-type second chromosomes. Newly eclosed adults from each of 109 different strains were assayed for DDC activity using the micro-diffusion assay described in Clark et al. (1978). The enzyme activity for each strain is expressed as a percentage of Canton-S activity. The positions in the distribution of the 4 strains selected for further study are indicated by the arrows.


WGM- 06, 19
WGM-65
WGM-40
Number of Strains
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
155
DDC activity (% Canton-S)

WGM-40, and WGM-65 were selected for further study because they fall at the two extremes of the distribution. Since the survey involved only one DDC activity determination, assays were repeated on young adults from these four strains. These determinations showed WGM-40 and WGM-65 to have slightly lower activity levels relative to Canton-S than is seen in Figure 6. However it is clear that all four strains are significantly different from Canton-S (R. B. Hodgetts personal communication, Table 3).

C. Genetic analysis

The structural gene for DDC lies on the second chromosome between the markers *hk* and *pr* (Wright et al., 1976). Experiments were performed on strains WGM-06, WGM-19, WGM-40, and WGM-65 to determine whether the element(s) responsible for the variation in DDC activity segregates with the second chromosome. The results, as described in Table 4, show this to be the case for strains WGM-06, WGM-40, and WGM-65 (R. B. Hodgetts, unpublished data). In contrast only a small amount of the variation in activity observed in WGM-19 is attributable to the second chromosome. For this reason no further experiments were carried out with this strain.

The crosses described in Figure 2 were designed to further localize the activity variation element(s) to the region between *hk* and *pr*. No results were obtained with strains WGM 06 or WGM 65 because the presence of recessive

Table 3

Extremes of Variation in Levels of DDC Activity in Natural Populations

Strain	DDC Activity † (units/mg live weight)	% Difference From Canton-S
WGM-40	4.16 ± 0.15	+19.9
WGM-65	3.79 ± 0.08	+ 9.2
Canton-S	3.47 ± 0.11	0.0
WGM-06	2.51 ± 0.10	-27.7
WGM-19	2.40 ± 0.08	-30.8

† Mean of 3 determinations ± 1 standard deviation

Table 4

Autosomal Segregation of Activity Variation

	DDC Activity (units/mg live weight)	
Strain	*+/Cy ; +/+*	+/+ ; +/Sb
WGM–40	3.83	4.91
WGM–65	3.25	4.25
WGM–06	3.00	2.43
WGM–19	3.00	2.73

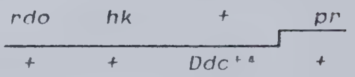
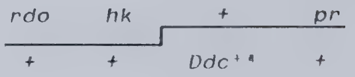
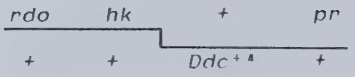
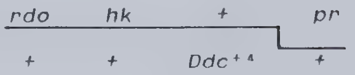
lethals made it impossible to generate homozygous recombinant chromosomes. The results for strain WGM 40 are summarized in Table 5. These results suggest that the element responsible for the variation lies between *hk* and *pr*. We have named this element Ddc^{+4}. To strengthen this conclusion we repeated this experiment using a Ddc^{+4} strain marked with *pr* which was generated in the first set of crosses, and a *hk* strain, as the 2 parents. The results of this second set of crosses support the initial conclusion (Table 6). After these experiments were completed, I became aware that the data in Tables 5 and 6 do not unambiguously place the Ddc^{+4} element between *hk* and *pr*. They are also consistent with a map position approximately 10 map units to the left of *hk*. Experiments designed to resolve this ambiguity are underway. This problem is examined in detail in the Discussion.

A standard mapping equation (Nash, 1963) was used to calculate the exact position of the variant element between the markers *hk* and *pr* given that it lies in this region. The map position = $Z+(a/a+b)x$, where Z is the map position of *hk*, x is the number of map units between *hk* and *pr*, a is the number of recombination events between *hk* and the variant element, and b is the number of events between the variant element and *pr*. Using the data in Tables 5 and 6 we have $Z=53.9$, $x=.6$, $a=2$, and $b=21$. Solving this equation gives a map position for Ddc^{+4} of 53.95. The 95% confidence interval for this map position is determined by using the equation:

Table 5

Genetic Mapping of DDC Overproduction in WGM-40

Parental Genotype	DDC Activity*	Phenotype
$\frac{rdo\quad hk\quad pr}{rdo\quad hk\quad pr}$	2.10	normal
$\frac{+\quad +\quad +}{+\quad +\quad +}$	3.83	overproducer

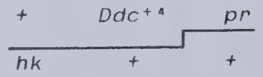
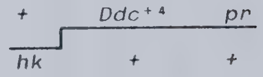
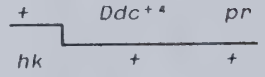
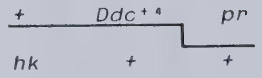
Recombinant Genotype	Number	DDC Activity*	Phenotype	Presumed Recombinational Event
$\frac{+\quad +\quad pr}{+\quad +\quad pr}$	7	3.53 ± 0.29	overproducer	
$\frac{+\quad +\quad pr}{+\quad +\quad pr}$	1	2.18	normal	
$\frac{rdo\quad hk\quad +}{rdo\quad hk\quad +}$	0	–	overproducer	
$\frac{rdo\quad hk\quad +}{rdo\quad hk\quad +}$	1	1.97	normal	

* DDC activity measured as units/mg live weight

Table 6

Genetic Localization of *Ddc*$^{+4}$ between *hk* and *pr* on Chromosome 2

Parental Genotype	DDC Activity*	Phenotype
$\frac{+\quad Ddc^{+4}\quad pr}{+\quad Ddc^{+4}\quad pr}$	4.04	overproducer
$\frac{hk\quad +\quad +}{hk\quad +\quad +}$	2.73	normal

Recombinant Genotype	Number	DDC Activity*	Phenotype	Presumed Recombinational Event
$\frac{hk\quad pr}{hk\quad pr}$	7	2.77 ± 0.32	normal	
$\frac{hk\quad pr}{hk\quad pr}$	0	–	overproducer	
$\frac{+\quad +}{+\quad +}$	1	2.53	normal	
$\frac{+\quad +}{+\quad +}$	6	4.37 ± 0.55	overproducer	

* DDC activity measured as units/mg live weight

location = $Z+p(x)$ where p represents the roots of the equation: $p^2\{(a+b)^2+\chi_1^2(a+b)\} - p\{2a^2+2ab+\chi_1^2(a+b)\} + a^2 = 0$. The confidence interval calculated in this way extends from map position 53.91 to 54.05. This corresponds well to the position of the gene for DDC as determined by Wright et al.(1976) (Figure 7).

D. Developmental characterization of *Ddc*$^{+4}$

The behavior of the *Ddc*$^{+4}$ variant throughout development was investigated by collecting *Ddc*$^{+4}$*pr* and Canton-S organisms at hatch, the 2/3 molt, and at pupariation and assaying DDC activity. The rate of development was the same for both strains (data not shown). As can be seen in Table 7 the variant is stage specific in its behavior. There is an overproduction of enzyme activity with respect to Canton-S at all stages except pupariation at which time there is a marked underproduction. This result is of special interest because pupariation is the only time during development that the appearance of DDC activity is closely related to the appearance of the insect steroid hormone ecdysterone (Kraminsky et al., 1980). Also this stage specific phenotype suggests that the variation in enzyme activity is a result of a change in the steady state level of enzyme and not a result of a change in specific activity.

To determine if the element responsible for the variation in activity at pupariation is in the immediate

Figure 7: Chromosomal location of the *Ddc*$^{+4}$ element(s). The map position of *Ddc*$^{+4}$ and the 95% confidence interval were determined as described in the text. The map position of *hk*, *Ddc*, and *pr* are from Wright et al. (1976).

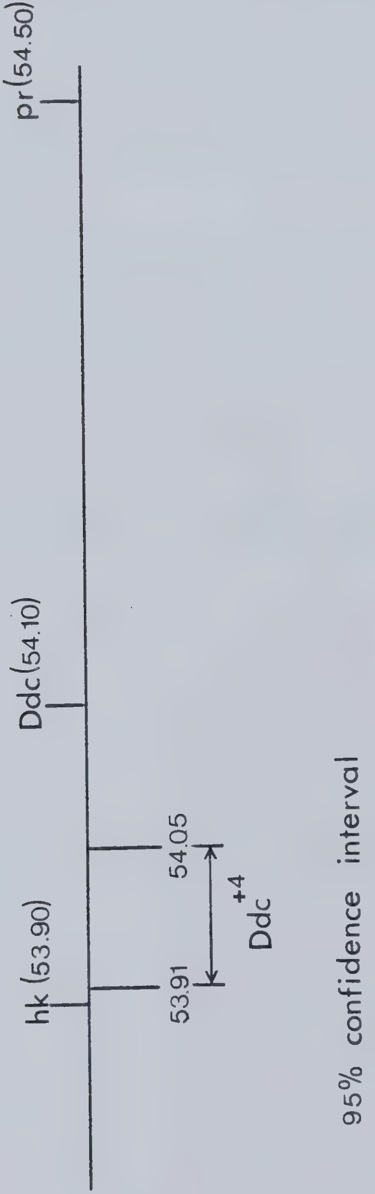

hk (53.90)
Ddc (54.10)
pr (54.50)
53.91
54.05
Ddc+4
95% confidence interval

Table 7

DDC Activity During Development of Canton-S and *Ddc*$^{+4}$ *pr* Strains

	DDC Activity (units/mg live weight) †			
Strain	Hatch	2/3 Molt	Pupariation	Eclosion
Ddc^{+4}	1.64±0.27	2.45±0.16	1.96±0.03	4.04±0.23
Canton-S	1.16±0.07	1.63±0.22	3.90±0.20	3.20±0.11
Ddc^{+4}:Canton-S	1.41	1.50	0.50	1.18

† Mean of 3 determinations ± 1 standard deviation

vicinity of the *Ddc* gene, the recombinants described in Table 6 were collected as white puparia and assayed for enzyme activity. This data is presented in Table 8. It is clear that the variation at pupariation is behaving the same genetically as the variation at eclosion and therefore may be determined by the same genetic change. However it is important to note that the variant chromosome was originally isolated from the wild and thus could easily carry several polymorphisms near the *Ddc* gene.

E. Sensitivity to dietary alpha methyl Dopa

Sensitivity to the drug αMD has been shown to be related in some complex way to the level of DDC activity (Wright et al.,1982). It was therefore of interest to measure the αMD sensitivity of *Ddc*$^{+4}$. Figure 8 shows that the variant is clearly less sensitive to the drug than Canton-S. The implications of this result are dealt with more fully in the Discussion section.

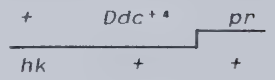
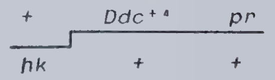
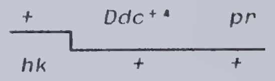
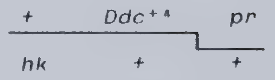
F. Immunotitration of DDC

To show that changes in enzyme activity reflect changes in the amount of enzyme, DDC activity was immunotitrated in adult and white prepupal extracts with a monospecific antiserum. The results for each titration were plotted, and the concentration of antiserum giving a 50% titration of enzyme activity determined from the curve. Each experiment was performed 3 times with a different extract each time.

Table 8

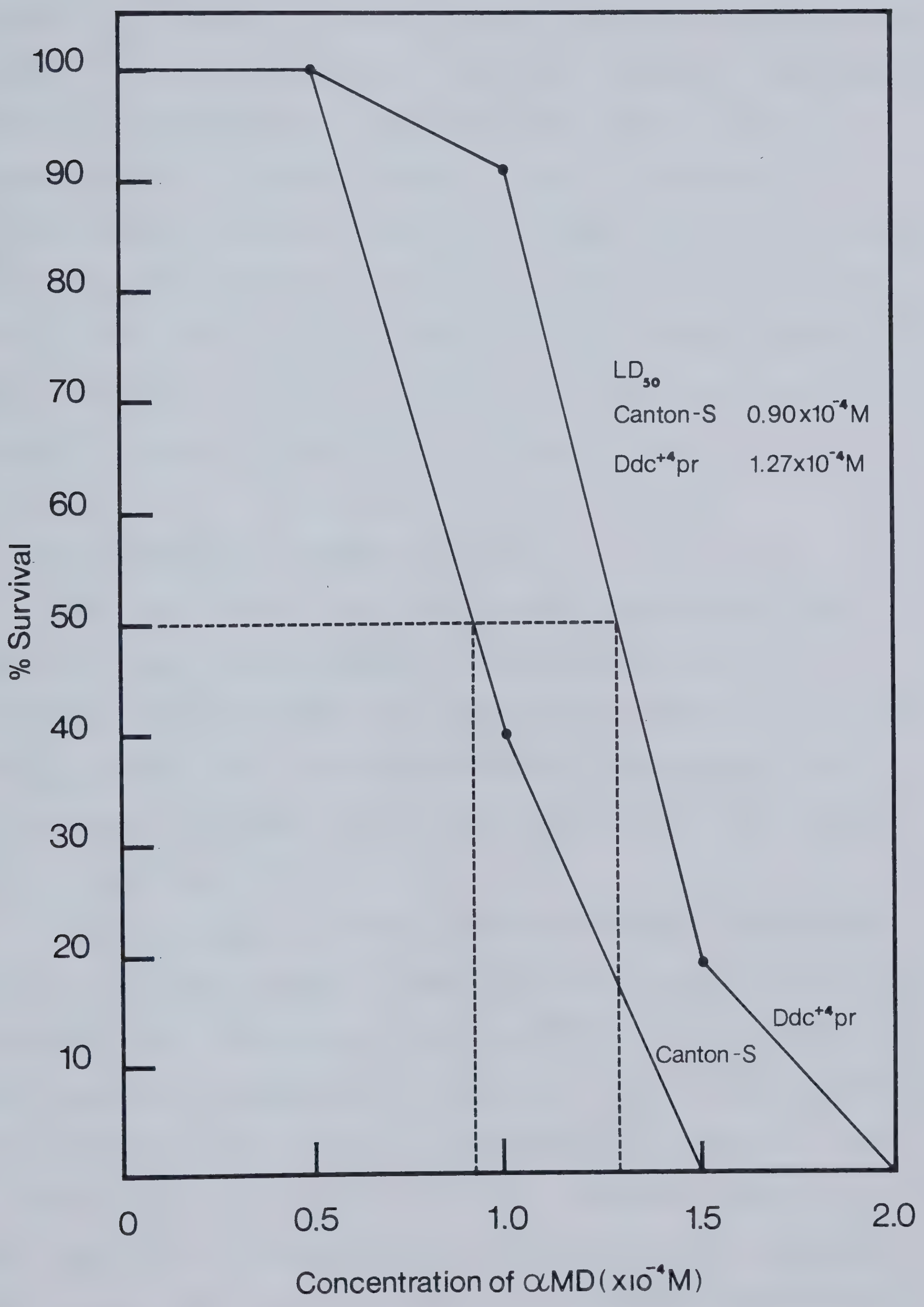
Genetic Localization of Underproduction in Puparia of *Ddc*$^{+4}$

Parental Genotype	DDC Activity*	Phenotype
$\frac{+\quad Ddc^{+4}\quad pr}{+\quad Ddc^{+4}\quad pr}$	2.00	underproducer
$\frac{hk\quad +\quad +}{hk\quad +\quad +}$	4.19	normal

Recombinant Genotype	Number	DDC Activity*	Phenotype	Presumed Recombinational Event
$\frac{hk\quad pr}{hk\quad pr}$	7	3.65 ± 0.48	normal	
$\frac{hk\quad pr}{hk\quad pr}$	0	–	underproducer	
$\frac{+\quad +}{+\quad +}$	1	3.50	normal	
$\frac{+\quad +}{+\quad +}$	6	2.51 ± 0.16	underproducer	

* DDC activity measured as units/mg live weight

Figure 8: Sensitivity to dietary α-methyl Dopa. Canton-S and *Ddc*$^{+4}$*pr* larvae were tested for survival on food supplemented with various concentrations of α-methyl Dopa as described in the Materials and Methods. The LD_{50} was determined from the graph.


100
90
80
70
60
50
40
30
20
10
% Survival
0
0.5
1.0
1.5
2.0
Concentration of αMD (x10⁻⁴M)
LD50
Canton-S 0.90x10⁻⁴M
Ddc⁺⁴pr 1.27x10⁻⁴M
Ddc⁺⁴pr
Canton-S

Typical titration curves are presented in Figures 9 and 10, and the means calculated from the three 50% titration values displayed in Table 9. In both white prepupae and adults the amount of enzyme in *Ddc*$^{+4}$*pr* relative to Canton-S measured in this way, corresponds closely to the relative amount of enzyme activity (compare Tables 7 and 9). When the concentration of antiserum giving a 50% titration is plotted versus enzyme activity for both strains and at both stages, the points form a line (Figure 11). This linear relationship indicates that differences in activity, between strains and stages, reflect differences in enzyme levels.

G. Electrophoretic analysis of DDC

DDC from Canton-S and *Ddc*$^{+4}$*pr* was compared on two different gel electrophoresis systems. *In vivo* ^{35}S methionine labelled DDC from both strains was run on an SDS polyacrylamide gel and visualized by fluorography. The fluorograph displayed in Figure 12 shows that the enzymes are of identical size.

In an attempt to detect possible charge differences between the two enzymes, crude extracts prepared from newly eclosed adult flies were run on cellulose acetate gels. In this case the enzymes were visualized using a radioiodinated antibody specific to DDC. No difference in migration was found between the Canton-S and *Ddc*$^{+4}$ enzymes (Figure 13). This method of enzyme detection also provides an estimate of CRM levels. The amount of CRM was not quantified but from

Figure 9: Immunotitration of DDC in white puparia. Monospecific anti-DDC antiserum was used to determine the amount of cross-reacting material (CRM) in Canton-S and *Ddc*$^{+4}$*pr* white puparia by immunotitration as described in the Materials and Methods. The concentration of antisera at 50% inactivation of enzyme was determined from the curve.

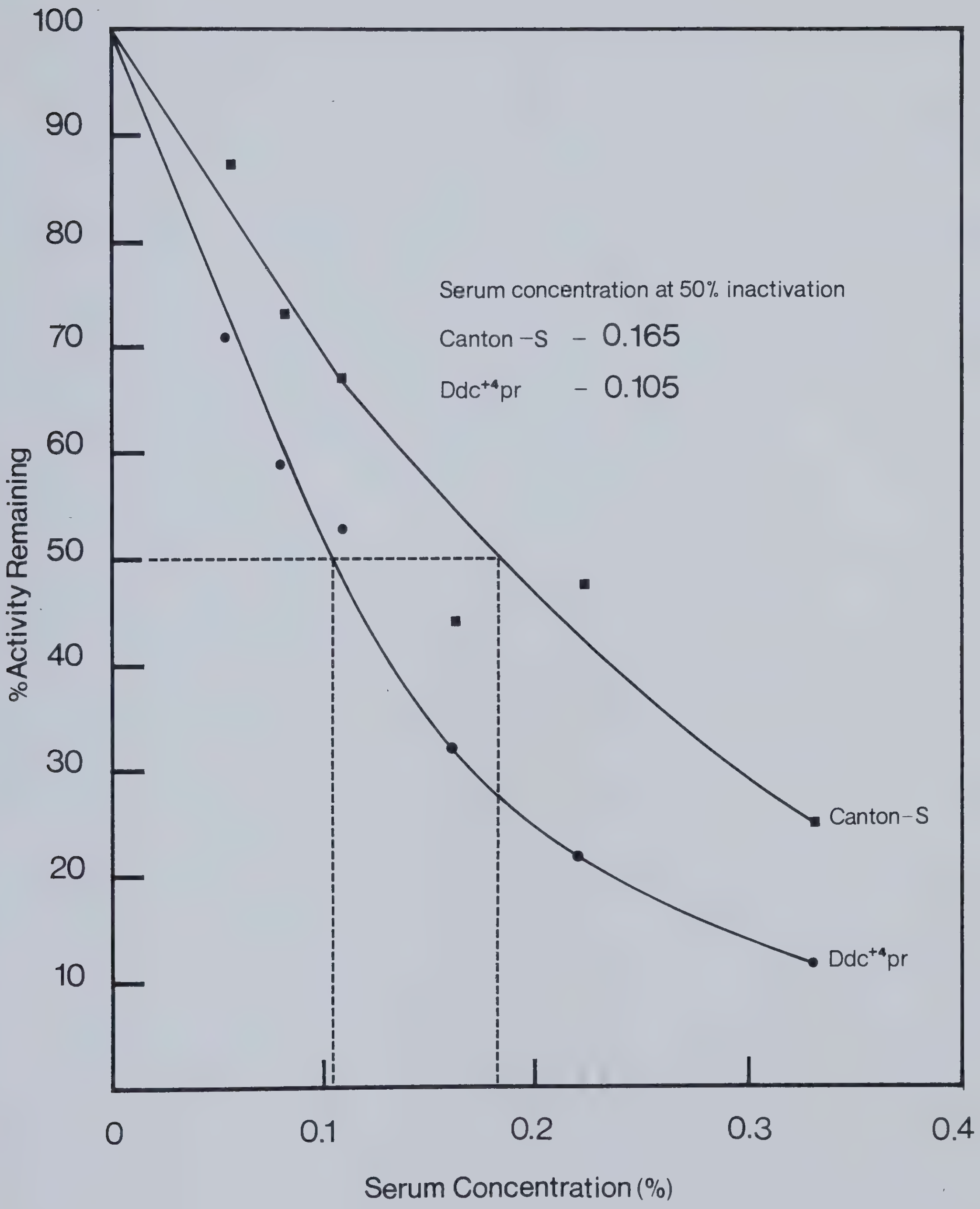

Serum concentration at 50% inactivation
Canton –S – 0.165
Ddc^{+4}pr – 0.105
Canton–S
Ddc^{+4}pr
% Activity Remaining
100
90
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
Serum Concentration (%)

Figure 10: Immunotitration of DDC in newly eclosed adults. Monospecific anti-DDC antiserum was used to determine the amount of CRM in Canton-S and *Ddc*$^{+4}$*pr* newly eclosed adults by immunotitration as described in the Materials and Methods. The concentration of antiserum at 50% inactivation was determined from the curve.

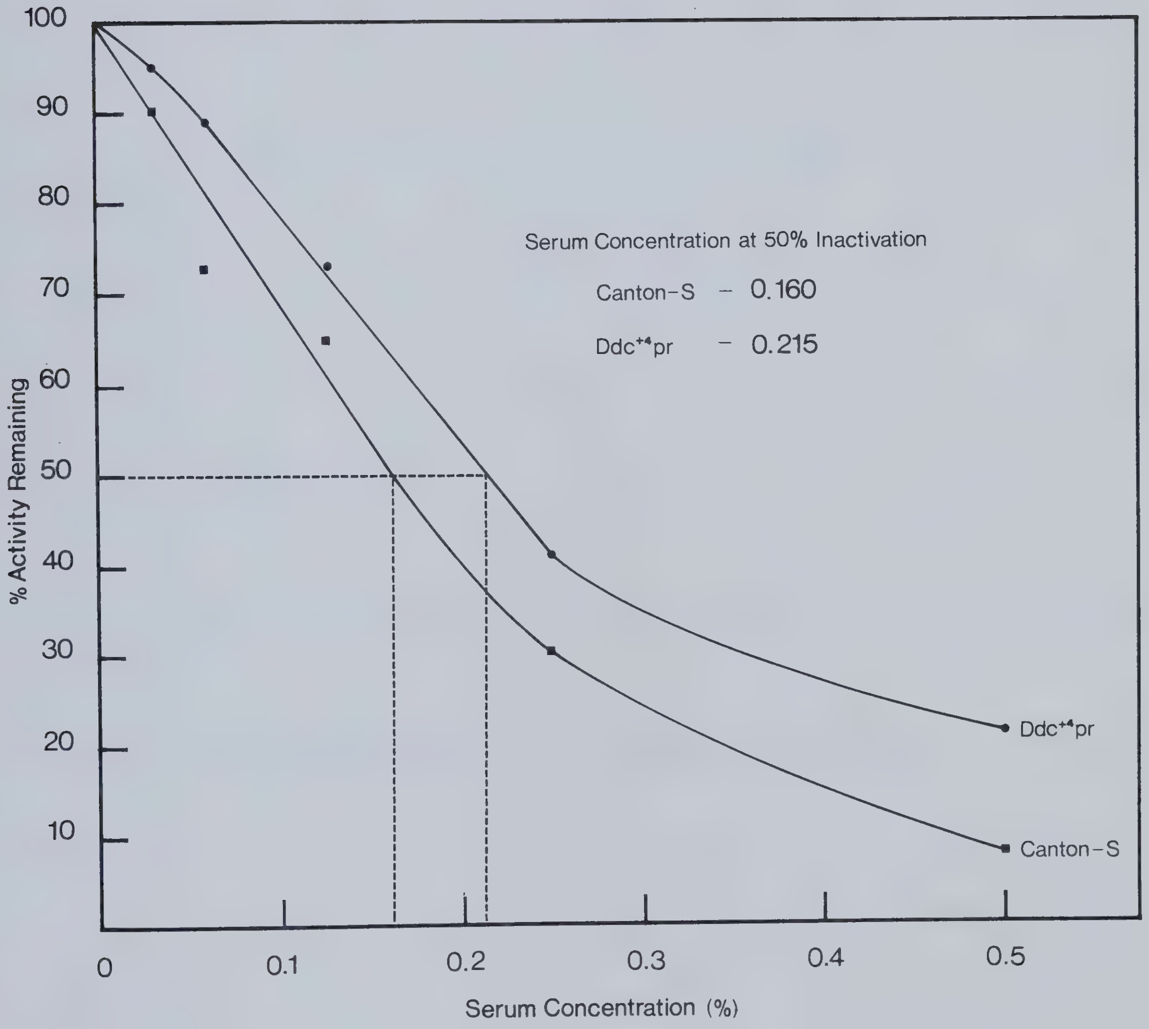

100
90
80
70
60
50
40
30
20
10
% Activity Remaining
0
0.1
0.2
0.3
0.4
0.5
Serum Concentration (%)
Serum Concentration at 50% Inactivation
Canton-S – 0.160
Ddc^{+4}pr – 0.215
Ddc^{+4}pr
Canton-S

Table 9

DDC Cross Reacting Material in Canton-S and *Ddc*$^{+4}$ *pr* Strains

	50% Titration Point (serum concentration in %)†	
Strain	Pupariation	Eclosion
Canton-S	0.193 ± 0.014	0.163 ± 0.020
Ddc^{+4} *pr*	0.105 ± 0.024	0.213 ± 0.012
Ddc^{+4} *pr*:Canton-S	0.540	1.310

† Mean of 3 determinations ± 1 standard deviation

Figure 11: The relationship between CRM levels and DDC activity levels in Canton-S and *Ddc*$^{+4}$*pr* strains. The level of CRM, as measured by immunotitration of DDC in Canton-S and *Ddc*$^{+4}$ *pr* extracts, was plotted against enzyme activity. CRM measurements, expressed as the antiserum concentration at 50% enzyme inactivation are found in Table 9. Enzyme activity levels are presented in Table 7. The line was extrapolated to the origin.

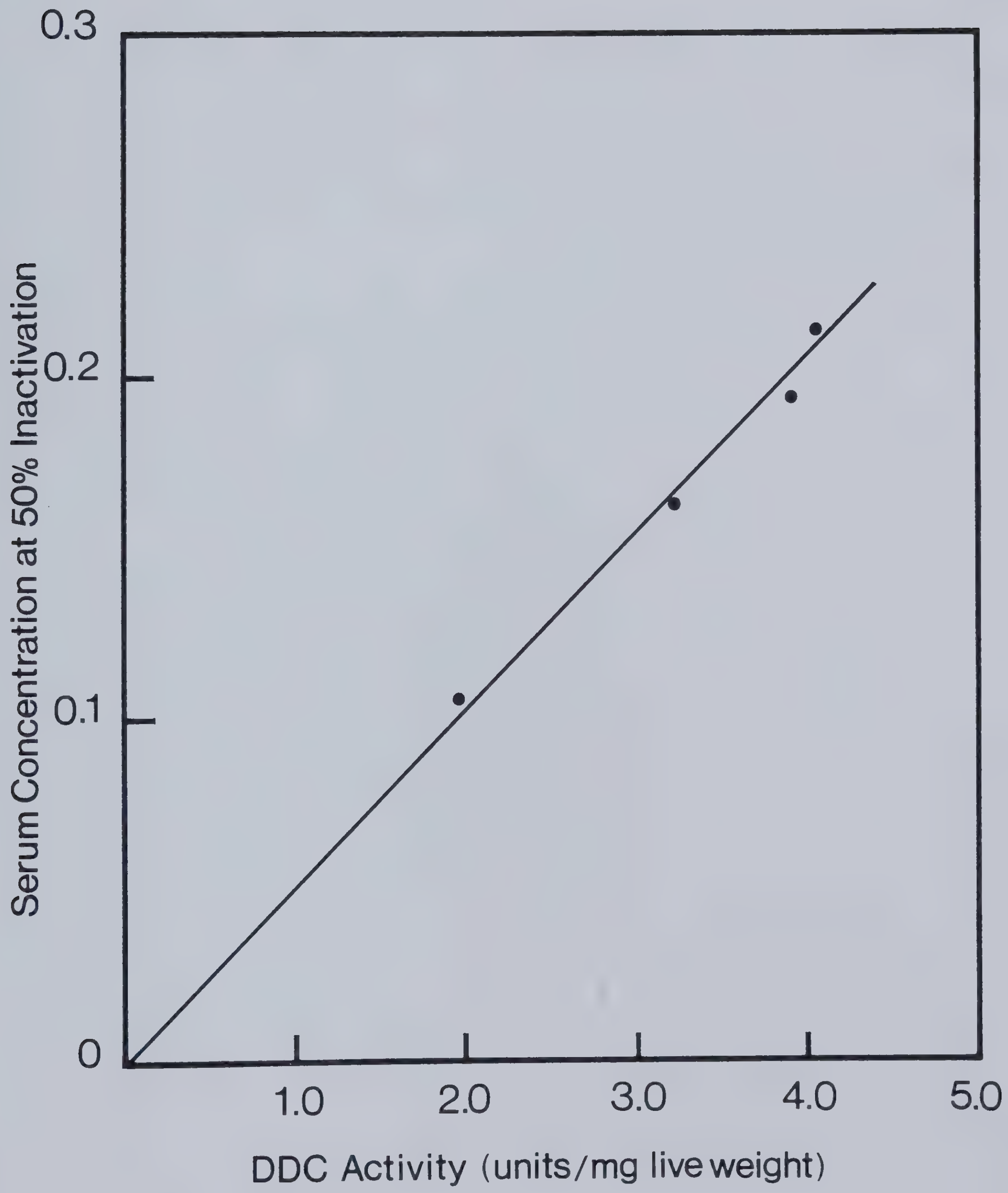

0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
Serum Concentration at 50% Inactivation
DDC Activity (units/mg live weight)

Figure 12: SDS polyacrylamide gel electrophoresis of immunoprecipitated DDC from Canton-S and *Ddc*$^{+4}$ strains. Larval proteins were radioactively labelled *in vivo* with ^{35}S methionine. DDC was immunoprecipitated from crude homogenates with purified anti-DDC IgG and *Staphylococcus aureus*. Labelled DDC was separated from IgG, electrophoresed on an SDS polyacrylamide gel and detected by fluorography. Slot 1 is DDC from Canton-S larvae; slot 2 is an approximately 1:1 mixture of extracts of Canton-S and *Ddc*$^{+4}$*pr* larvae; slot 3 is DDC from *Ddc*$^{+4}$*pr* larvae. The horizontal line indicates the position of the IgG heavy chain protein. This protein comigrates with DDC.

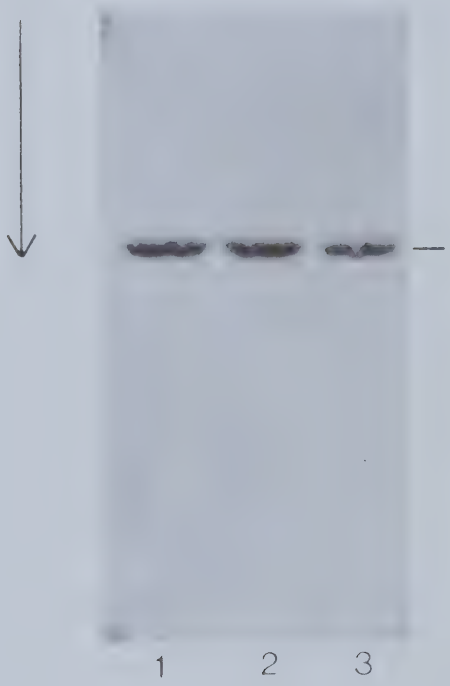
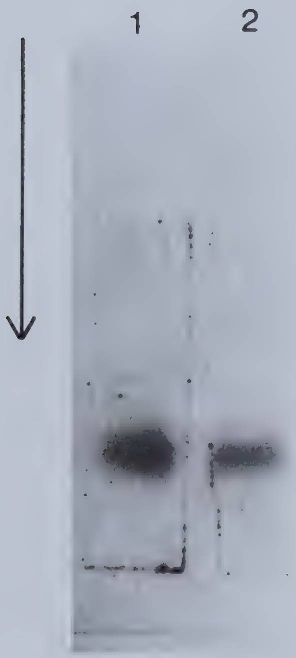

1
2
3

Figure 13: Cellulose acetate gel electrophoresis of DDC from Canton-S and *Ddc*$^{+4}$ strains. Crude homogenate prepared from newly eclosed adults was electrophoresed on Cellogel-500 strips (Chemetron). DDC was precipitated in the gel with ^{125}I labelled anti-DDC IgG and visualized by autoradiography. Slot 1 contains *Ddc*$^{+4}$ homogenate and slot 2 Canton-S homogenate.


1
2

visual examination alone it is clear that the *Ddc*$^{+4}$*pr* crude homogenate contains more CRM than does the Canton-S crude homogenate. This observation is consistent with the immunotitration experiments described earlier.

H. DNA organization in the region of the *Ddc* gene

The availability of cloned genomic DNA (Hirsh and Davidson, 1981) facilitated the analysis of DDC specific transcripts and genomic DNA organization. These clones were obtained from a Canton-S library constructed by J. Lauer (Maniatis et al., 1978). The clones, along with information on the location of restriction endonuclease sites, were kindly supplied by Cliff Brunck and Jay Hirsh. Figure 14 provides restriction site and transcript information for a 10 Kb region containing the *Ddc* gene (Hirsh and Davidson, 1981; this study). Table 10 lists the plasmids used in this study along with their derivation.

I. Extraction of Poly A$^+$ RNA

The phenol-chloroform RNA extraction procedure was used to extract RNA from newly hatched first instar larvae, wandering third instar larvae, and newly eclosed adults. Wandering third instar larvae were used instead of white prepupae because it is easier to collect quantities of larvae sufficient for RNA extraction.

In order to minimize variability in the efficiency of extraction, Canton-S and *Ddc*$^{+4}$ RNA were extracted in

Figure 14: Restriction map of DNA in the *Ddc* region of Canton-S. Information on the location of restriction sites was obtained from J. Hirsh (personal communication) and this study. Restriction sites for the enzymes Pst I, Bgl II, and Eco RV have been mapped only in the region bound by the Bam HI sites at coordinates 0.7 to 8.2. The approximate location of the *Ddc* transcription unit is indicated above the restriction site map (J. Hirsh, personal communication). Exon sequences are represented by straight lines and intron sequences by cross hatched boxes.

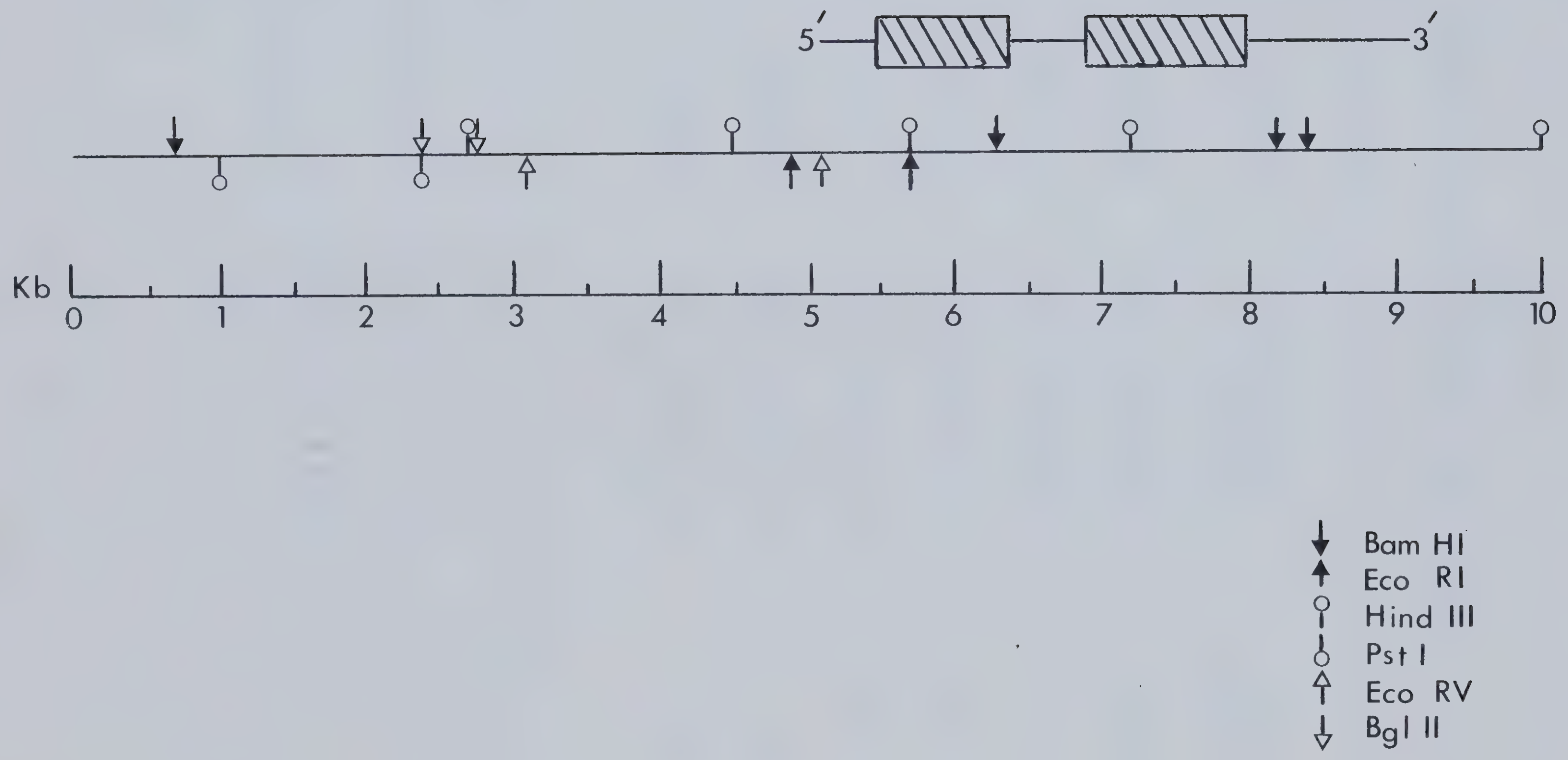

5′
3′
Kb
0
1
2
3
4
5
6
7
8
9
10
Bam HI
Eco RI
Hind III
Pst I
Eco RV
Bgl II

Table 10

Plasmids Containing *Ddc* Gene Sequence

Plasmid Designation	Vector	*Drosophila* Insert	Source of *Drosophila* DNA
pDDC–1	pBR322	Bam HI/Bam HI (0.7 - 6.3)	Canton–S
pDDC–4	pBR322	Bam HI/Bam HI (6.3 - 8.2)	Canton–S
pDDC–9	pBR322	Bam HI/Eco RI† (0.7 - 10.4)	Canton–S
pDDC–10	pUC8	Eco RI/Eco RI† (5.7 - 10.4)	Canton–S
pDDC40–1	pBR322	Bam HI/Bam HI (6.3 - 8.2)	*Ddc*$^{+4}$
pDDC40–2	pBR322	Bam HI/Bam HI (0.7 - 6.3)	*Ddc*$^{+4}$

All Canton–S sequences were gifts from Drs. C. Brunck and J. Hirsh

† This Eco RI site is an artificial restriction site created by cloning

parallel under exactly the same conditions and using the same reagents. Since the organisms were homogenized in buffer containing 2% w/v SDS, both nuclear and cytoplasmic RNA was extracted. Total RNA from a particular pair of extractions was chromatographed on the same oligo-dT cellulose column. Even after oligo-dT chromatography ribosomal RNA is a significant contaminant in the poly A^+ RNA preparation. Since I was interested in comparing the amount of DDC transcript present in Canton-S and *Ddc*$^{+4}$ poly A^+ RNA, it was important to ensure that this contamination was equivalent in both samples. Towards this end, poly A^+ RNA samples were rechromatographed on oligo-dT cellulose repeatedly until no RNA was detected in the wash fractions. Usually this required 2 passages over the column. Representative RNA yields at all three stages are summarized in Table 11.

J. The level of DDC transcript in *Ddc*$^{+4}$ and Canton-S

The object of these experiments was to compare the steady state levels of DDC specific transcripts in Canton-S and *Ddc*$^{+4}$ strains. Differences in the amount of DDC transcript in *Ddc*$^{+4}$ concurrent with the differences in enzyme activity described above would suggest that the observed variation in the level of gene expression is a result of an altered rate of transcription or a change in the processing or stability of transcripts.

Table 11

RNA Extraction Yields

	Live Weight (gm)	Total RNA (μg)	Poly A$^+$ RNA (μg)
First Instar			
Canton–S	0.5	2,180	78
Ddc^{+4} *pr*	0.5	2,600	72
Third Instar			
Canton–S	2.9	24,600	190
Ddc^{+4} *pr*	3.6	27,200	172
Adults			
Canton–S	0.3	1,980	40
Ddc^{+4}	0.3	1,820	29

Poly A^+ RNA from Canton-S and *Ddc*$^{+4}$*pr* newly hatched first instar larvae, wandering third instar larvae, and newly eclosed adults was analyzed on northern blots. At least 2 blots were prepared for each stage and each blot in turn contained 4 slots of Canton-S RNA and 4 slots of *Ddc*$^{+4}$ RNA. Blots were probed with nick-translated 4.8 Kb Eco RI fragment (coordinate 5.7 to 10.5 on Figure 14) or 1.9 Kb Bam HI fragment (6.3 to 8.2). The signal intensity in each slot was quantified as described in the Materials and Methods and the result expressed in terms of arbitrary units/μg RNA loaded. Comparisons can be made only between samples on the same blot because of variability in blotting and hybridization. For the purposes of these experiments the important parameter is the ratio of *Ddc*$^{+4}$ to Canton-S signal on each blot.

As can be seen in Figures 15, 16, and 17 and summarized in Table 12, the relative amount of DDC transcript in *Ddc*$^{+4}$ closely parallels the relative amount of DDC activity at hatch, late third instar and at eclosion. At both hatch and eclosion there is between 40% and 50% more DDC transcript in the variant while in late third instar, *Ddc*$^{+4}$ has only 50% the amount of transcript present in Canton-S RNA preparations.

Figure 15: Comparison of DDC transcript levels in Canton-S and *Ddc*$^{+4}$ strains in early first instar larvae. One µg of poly A^{+}RNA from Canton-S and *Ddc*$^{+4}$*pr* newly hatched first instar larvae was run on a 1.25% agarose gel in the presence of 3% v/v formaldehyde. The RNA was blotted onto nitrocellulose and the blot probed with nick-translated 1.9 Kb Bam HI fragment (6.3 to 8.2, Figure 14). Slots 1, 3, 5, and 7 contain Canton-S RNA while slots 2, 4, 6, and 8 contain *Ddc*$^{+4}$*pr* RNA. Yeast ribosomal RNA was run as a molecular weight standard and visualized with ethidium bromide.

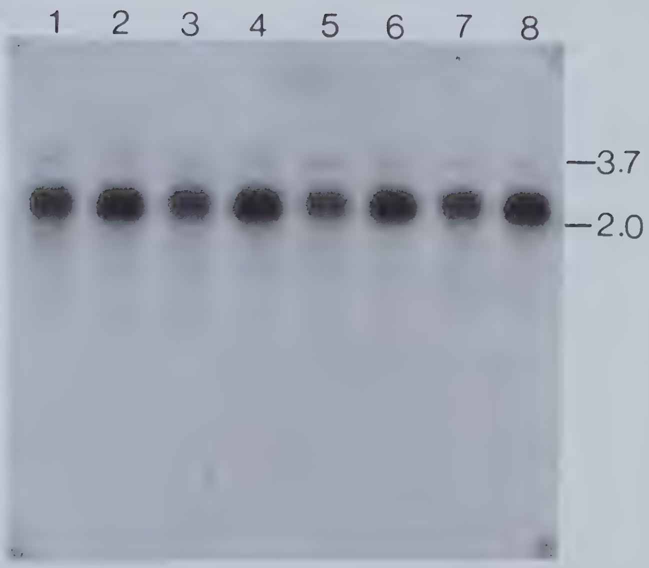

1
2
3
4
5
6
7
8
3.7
2.0

Figure 16: Comparison of DDC transcript levels in Canton-S and *Ddc*$^{+4}$ strains in wandering third instar larvae. One μg of Canton-S and *Ddc*$^{+4}$*pr* RNA was electrophoresed, blotted onto nitrocellulose, and probed as in Figure 15. Slots 1, 3, 5, and 7 contain Canton-S RNA and slots 2, 4, 6, and 8 contain *Ddc*$^{+4}$*pr* RNA.

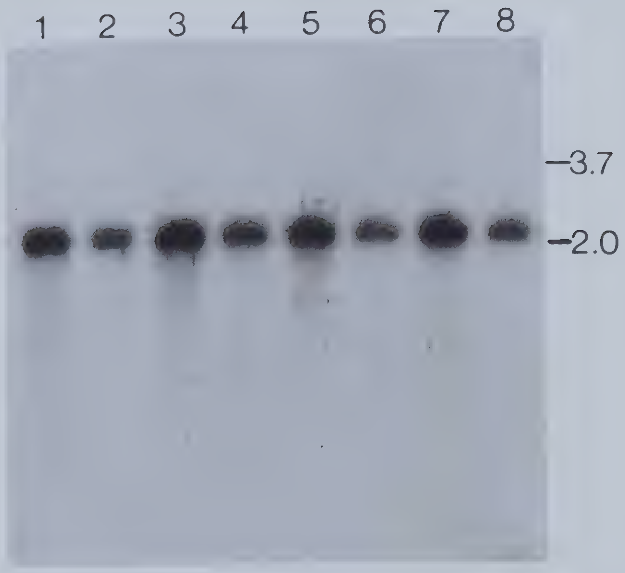

1
2
3
4
5
6
7
8
3.7
2.0

Figure 17: Comparison of DDC transcript levels in Canton-S and *Ddc*$^{+4}$ strains in newly eclosed adults. Northern analysis was performed on adult poly-A^{+} RNA from Canton-S and *Ddc*$^{+4}$ as described for Figure 15 except that 0.8 μg of RNA/slot was loaded and the blot was probed with nick-translated plasmid pDDC-10. Slots 1, 3, 5, and 7 contain Canton-S RNA and slots 2, 4, 6, and 8 contain *Ddc*$^{+4}$ RNA.

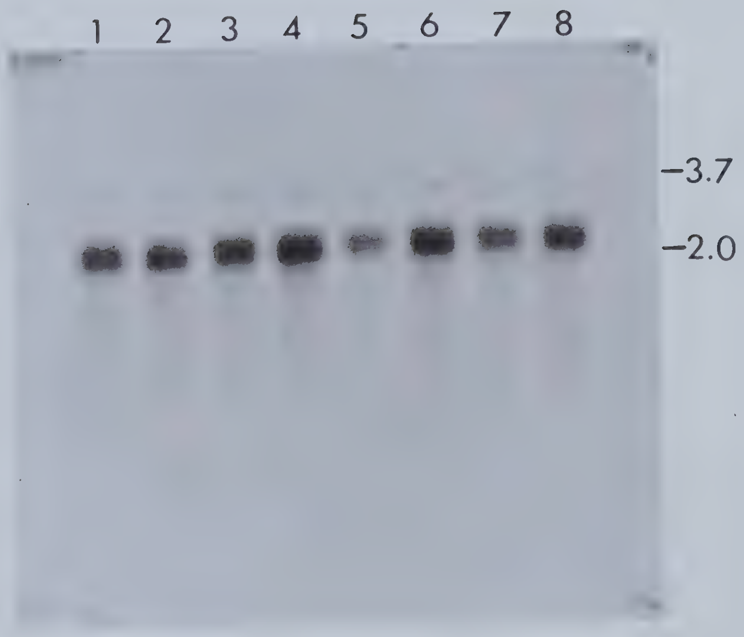

1
2
3
4
5
6
7
8
3.7
2.0

Table 12

Comparison of DDC Transcript Levels in Canton-S and *Ddc*$^{+4}$

	Mean units/μg RNA†		
Strain	**Hatch**	**Pupariation**	**Eclosion**
Canton-S	52 ± 7.1	54 ± 9.7	124 ± 37
Ddc^{+4}	74 ± 6.1	28 ± 5.2	184 ± 27
Ddc^{+4}:Canton-S	1.40	0.52	1.48

† Mean of 4 determinations ± the standard deviation. Comparisons can only be made within a stage since units are arbitrary and not normalized for experimental variation (for example, probe specific activity, autoradiograph exposure).

K. Cloning the *Ddc* region of *Ddc*$^{+4}$

In order to analyze the *Ddc*$^{+4}$ gene in detail it was necessary to construct a recombinant DNA library using a *Ddc*$^{+4}$ strain as a source of DNA. The details of library construction are described in the Materials and Methods. The library contained a total of 170,000 independent plaques before amplification. This represents approximately 1.3×10^5 plaques/μg of *Drosophila* DNA. After amplification the library titer was 1.5×10^6 pfu/ml.

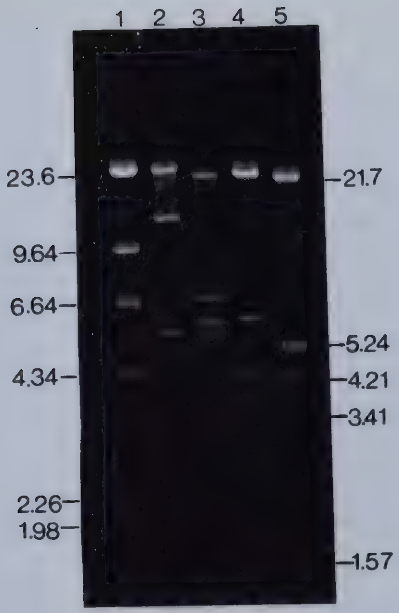
The *Drosophila* DNA fragments used to construct the λ1059:*Ddc*$^{+4}$ library were between 15 and 20 Kb in length. In order to be 99% confident of cloning a particular DNA sequence approximately 25,000 recombinant bacteriophage containing fragments of this size need to be screened (Clarke and Carbon, 1976). To maximize the chances of detecting a *Ddc* containing bacteriophage, 50,000 plaques were grown on strain Q 358 and screened with agarose gel purified 1.9 Kb Bam HI fragment (6.3 to 8.2 on Figure 14). A total of 7 strongly hybridizing plaques were picked and rescreened after plating onto strain Q 359.

Restriction analysis of bacteriophage DNA showed that the 7 recombinants were of 3 types. The first type, of which there are 2 identical representatives, λ15 and λ4, contained a *Drosophila* insert which spans the *Ddc* region. Figures 18 and 19 show a restriction analysis and Southern blot of λ15. The second type is a λ1059 recombinant with a *Drosophila* insert which hybridizes strongly to the 1.9 Kb Bam HI

Figure 18A: Restriction analysis of λ15. Approximately 1 μg/slot of λ15 DNA was restricted with endonuclease and electrophoresed on a 0.8% agarose gel. Slots 2, 3, and 4 contain λ15 DNA restricted with Bam HI, Eco RI and Hind III respectively. Slots 1 and 5 contain λCI857 DNA restricted with Hind III and Eco RI plus Hind III respectively.

Figure 18B: Restriction map of the λ15 insert. The *Drosophila* insert contained in λ15 is illustrated above the restriction map of the *Ddc* region. The two ends of the insert sequence fall within the dotted lines at each extreme.

A


1
2
3
4
5
23.6
9.64
6.64
4.34
2.26
1.98
21.7
5.24
4.21
3.41
1.57

B

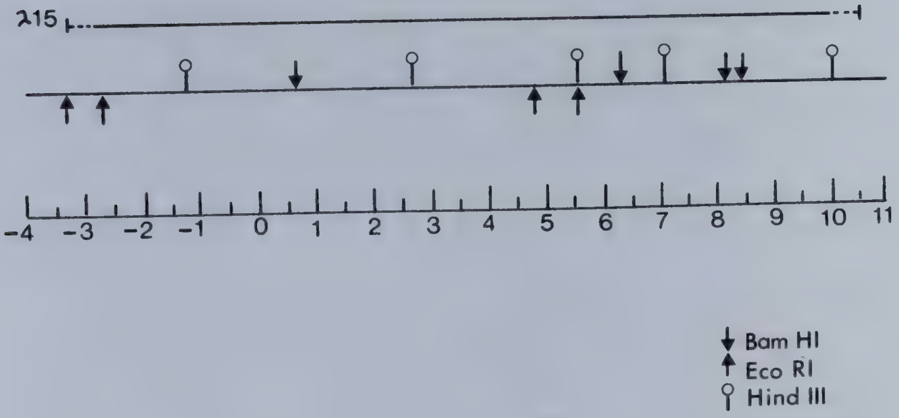
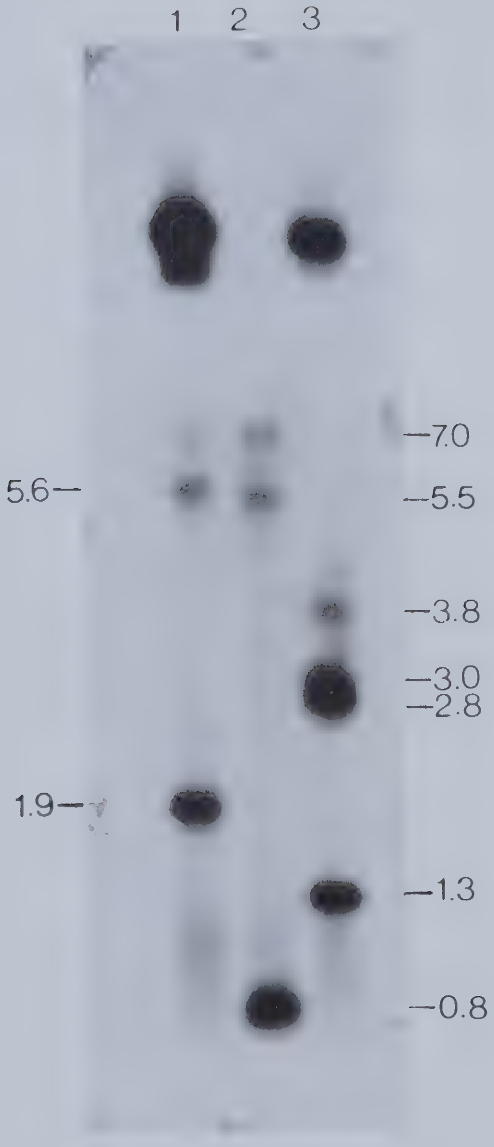

λ15
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
Bam HI
Eco RI
Hind III

Figure 19: Southern analysis of λ15. The DNA in the gel displayed in Figure 18A was blotted onto nitrocellulose and the blot probed with nick-translated pDDC-9. This plasmid contains sequences from coordinates 0.7 to 10.4 (Figure 14) and hence spans the entire *Ddc* region. Slots 1, 2, and 3 correspond to slots 2, 3, and 4 in Figure 18A.


1
2
3
5.6
1.9
7.0
5.5
3.8
3.0
2.8
1.3
0.8

fragment but has a restriction pattern completely unlike that of DNA in the *Ddc* region. There were 3 identical examples of this type. The third type of bacteriophage is similar to the second type in that endonuclease restriction generates no familiar restriction fragments. In addition this bacteriophage seems to have undergone a rearrangement which introduces an Eco RI site in the left arm of the phage (data not shown).

L. The identification of DNA sequence polymorphisms in and adjacent to the *Ddc* gene of *Ddc*$^{+4}$

The results described in preceeding sections show that *Ddc*$^{+4}$ strains carry a mappable genetic polymorphism which alters the steady state level of DDC transcript and ultimately the amount of DDC enzyme activity. In an attempt to further characterize this polymorphism the DNA sequence organization of the *Ddc* region in *Ddc*$^{+4}$ was compared to that of Canton-S by examination of genomic Southern blots and by doing careful restriction analysis of cloned *Ddc*$^{+4}$ DNA.

A total of 5 differences between *Ddc*$^{+4}$ and Canton-S *Ddc* DNA were observed on genomic Southern blots. These are summarized in Figure 20 and Table 13. High molecular weight DNA was isolated from embryos, restricted with a variety of restriction enzymes, electrophoresed on an agarose gel, blotted onto nitrocellulose and probed with one of several nick-translated DNA probes. In addition to Canton-S and *Ddc*$^{+4}$ samples, restriction digests of DNA extracted from

Figure 20: The location of DNA polymorphisms in the *Ddc* region observed by comparison of *Ddc*$^{+4}$ and Canton-S DNA on genomic Southern blots. Five differences (a to e) were observed on Southern blots (Figures 21 and 22). The location of each polymorphism is shown below the restriction map. A description of these differences is found in Table 13. The location of the *Ddc* transcription unit is indicated as in Figure 14.

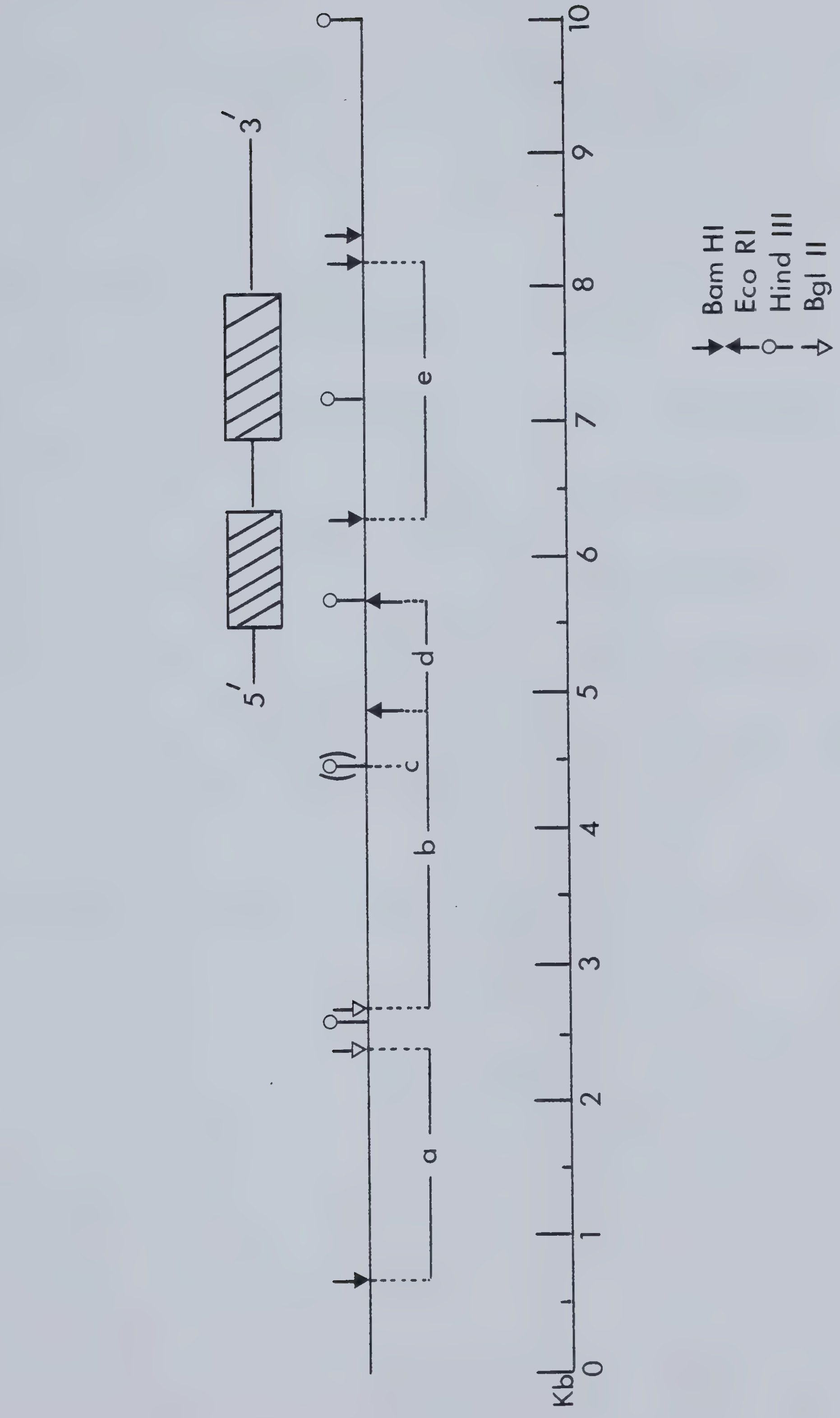

5'
3'
(?)
a
b
c
d
e
Kb
0
1
2
3
4
5
6
7
8
9
10
Bam HI
Eco RI
Hind III
Bgl II

Table 13

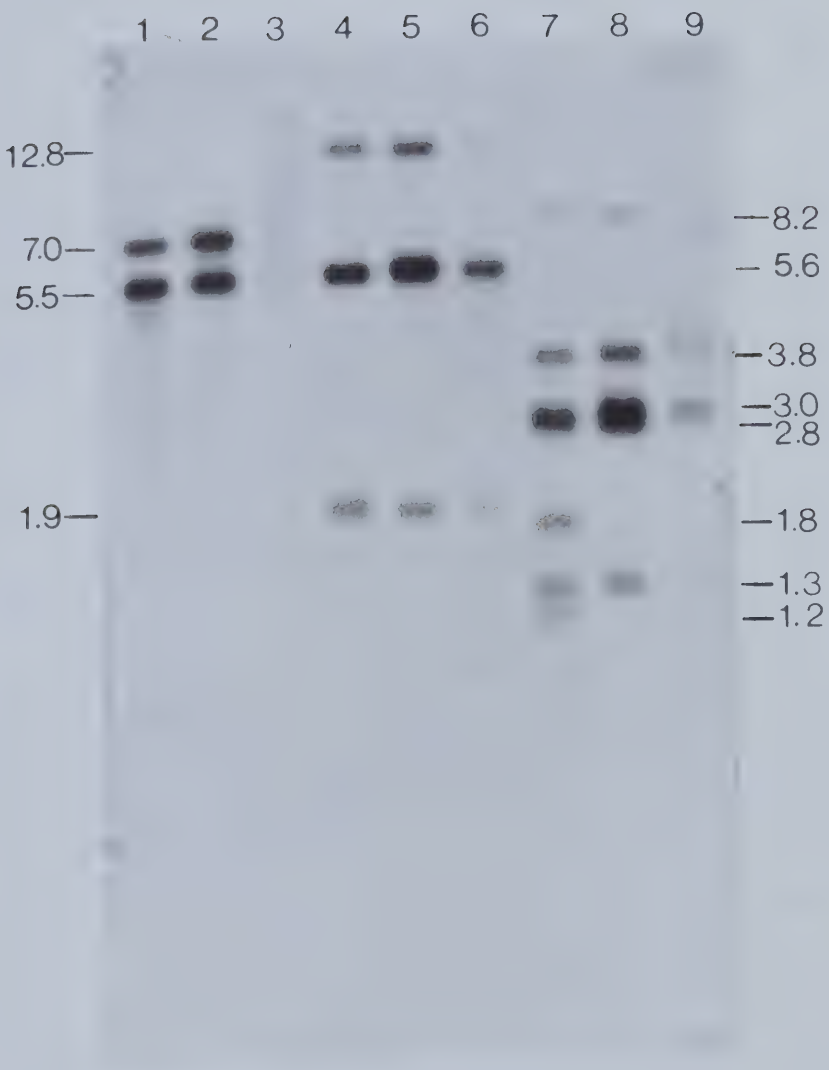
Restriction Fragment Polymorphisms Revealed by Southern Analysis of *Ddc*$^{+4}$ and Canton-S

Polymorphism	Restriction Fragment	Change in Fragment Size
a	Bam HI/Bgl II (0.7 - 2.3)	<100 nucleotide increase
b	Bgl II/Eco RI (2.7 - 4.9)	<100 nucleotide increase
c	—	loss of Hind III site at 4.5
d	Eco RI/Eco RI (4.9 - 5.7)	<100 nucleotide increase
e	Bam HI/Bam HI (6.3 - 8.2)	<100 nucleotide decrease

CyO/Df130 flies were included on these blots. Since *Df130* is a deficiency which removes the *Ddc* gene, this DNA contains *Ddc* sequence from the *CyO* chromosome only. The *CyO* gene for DDC is expressed at a similar level to the Canton-S gene (data not shown). Any polymorphism common to both the *Ddc*$^{+4}$ DNA and the *CyO* DNA is therefore not likely to be responsible for the variant *Ddc*$^{+4}$ phenotype.

Figure 21 shows an autoradiograph of a Southern blot probed with nick-translated plasmid pDDC-9. This plasmid contains *Drosophila* DNA spanning the entire *Ddc* gene plus approximately 1 Kb of 3' flanking sequence and 4 Kb of 5' flanking sequence (0.7 to 10.9). Both the Eco RI and Bam HI digests on this blot show that there are no major DNA rearrangements in the *Ddc*$^{+4}$ DNA. However this autoradiograph does reveal 2 differences between Canton-S and *Ddc*$^{+4}$. The Hind III restriction site present at coordinate 4.5 (Figure 14) in Canton-S is absent in *Ddc*$^{+4}$. This difference is called polymorphism c on Figure 20. Restriction of *CyO* DNA with Hind III (slot 9) shows that this site is absent in *CyO* as well. Slot 9 in Figure 21 also demonstrates additional differences in the *CyO* restriction pattern as compared to both Canton-S and *Ddc*$^{+4}$ restriction patterns. It appears that the Hind III site at coordinate 10 has been replaced by a site approximately 1.2 Kb further 3'. Consequently the 2.8 Kb Hind III fragment has been replaced by a 4.0 Kb fragment. The second *Ddc*$^{+4}$ polymorphism visible in this Figure (polymorphism e) is a small decrease (less than 100

Figure 21: Southern analysis of the *Ddc* region in Canton-S, *Ddc*$^{+4}$*pr*, and *Df130 cn bw/CyO* strains (I). High molecular weight DNA was isolated from embryos in the case of Canton-S and *Ddc*$^{+4}$*pr*, and from adults for *Df130 cn bw/CyO*, restricted with endonuclease, and electrophoresed on a 0.8% agarose gel. The DNA was blotted onto nitrocellulose and the blot probed with nick-translated pDDC-10. Slots 1, 4, and 7 contain Canton-S DNA; slots 2, 5, and 8 contain *Ddc*$^{+4}$*pr* DNA; and slots 6 and 9 contain *Df130 cn bw/CyO* DNA. The DNA in slots 1 and 2 was restricted with Eco RI, in slots 4, 5, and 6 with Bam HI, and in slots 7, 8, and 9 with Hind III.


1
2
3
4
5
6
7
8
9
12.8
7.0
5.5
1.9
8.2
5.6
3.8
3.0
2.8
1.8
1.3
1.2

nucleotides) in the size of the 1.9 Kb Bam HI fragment (6.3 to 8.2) from *Ddc*$^{+4}$ when compared to either the Canton-S or the *CyO* counterpart.

The autoradiograph displayed in Figure 22 reveals 3 additional differences. This blot has been probed with plasmid pDDC-1 which contains *Drosophila* DNA from coordinate 0.7 to 6.3. Polymorphism d is a small increase (less than 100 nucleotides) in the size of the .8 Kb Eco RI fragment (4.9 to 5.7). Polymorphism b is a small decrease (less than 100 nucleotides) in the size of the 2.3 Kb Eco RI/Bgl II fragment (2.7 to 4.9) and polymorphism a is a small increase (less than 100 nucleotides) in the size of the 1.7 Kb Bam HI/Bgl II fragment (0.7 to 2.4). Polymorphism a, or one similar to it at this level of resolution is present in *CyO* DNA as well. Polymorphisms b and d are unique to *Ddc*$^{+4}$ among these strains.

Restriction analysis of cloned *Ddc*$^{+4}$ sequences produced similar but not identical findings to those described above. In order to facilitate analysis of the *Ddc*$^{+4}$ DNA contained in λ15, 2 Bam HI fragments, the 1.9 Kb (6.3 to 8.2) and the 5.6 Kb (0.7 to 6.3) from λ15 were inserted into the Bam HI restriction site of the plasmid pBR322. These 2 plasmids were named pDDC 40-1 and pDDC 40-2 respectively. Their structures are described in Figure 23. The 2 plasmids containing analogous Canton-S fragments are also described in this Figure.

Figure 22: Southern analysis of the *Ddc* region in Canton-S, *Ddc*$^{+4}$*pr*, and *Df130 cn bw/CyO* strains (II). Southern analysis was performed as in Figure 21 except that the blot was probed with plasmid pDDC-1. Slots 1, 4, and 7 contain Canton-S DNA; slots 2, 5, and 8 contain *Ddc*$^{+4}$ *pr* DNA; and slots 3, 6, and 9 *Df130 cn bw/CyO* DNA. The DNA in slots 1, 2, and 3 was restricted with Eco RI and Bam HI; in slots 4, 5, and 6 with Eco RI and Bgl II, and slots 7, 8, and 9 with Bam HI and Bgl II.

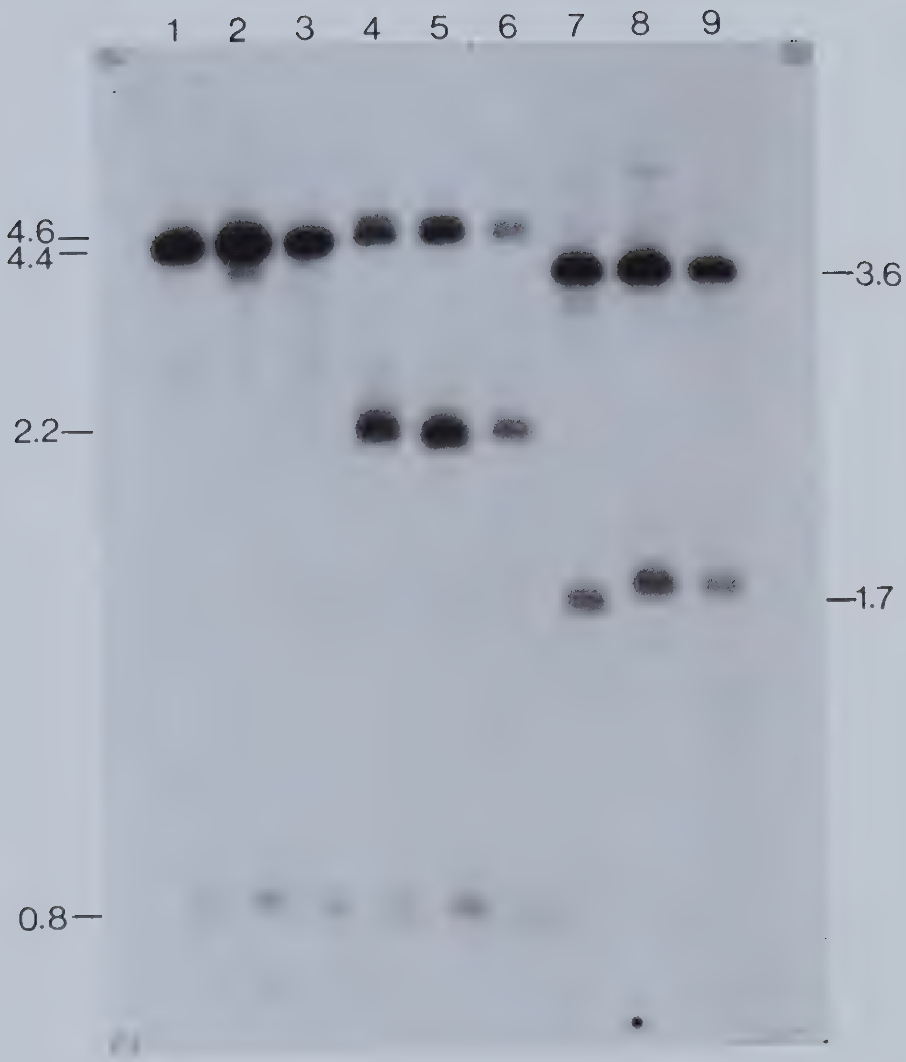
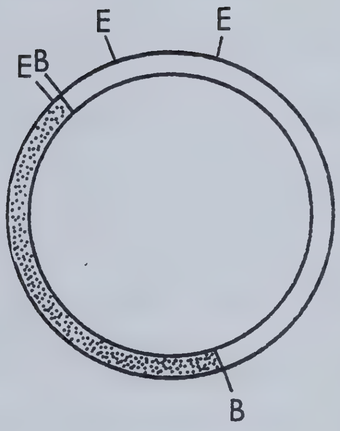
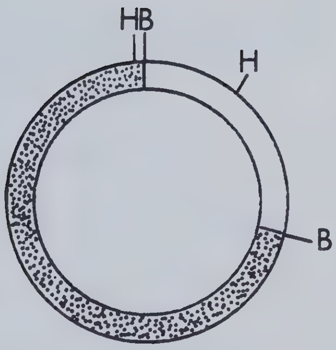

1
2
3
4
5
6
7
8
9
4.6
4.4
2.2
0.8
3.6
1.7

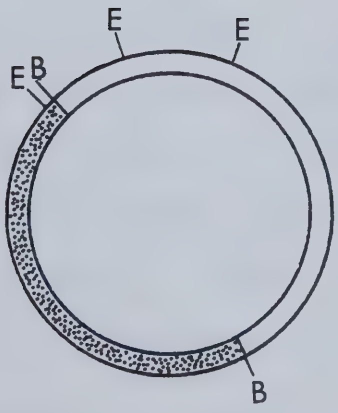
Figure 23: Description of recombinant plasmids containing DNA from the *Ddc* region of *Ddc*$^{+4}$ and Canton-S strains. Plasmids pDDC40-1 and pDDC40-2 were constructed by inserting *Drosphila* Bam HI fragments from λ15 into the Bam HI site of pBR322. pDDC40-1 contains the 1.9 Kb Bam HI fragment (6.3 to 8.2, Figure 14) and pDDC40-2 contains the 5.6 Kb Bam HI fragment (0.7 to 6.3). pDDC-1 is identical to pDDC40-2 except that the *Drosophila* fragment is derived from Canton-S. pDDC-4 contains the Canton-S 1.9 Kb Bam HI fragment. This fragment has been inserted in the opposite orientation relative to pDDC40-1. Eco RI (E) and Bam HI (B) restriction sites are indicated on pDDC40-2 and pDDC-1. Hind III (H) and Bam HI sites are shown on pDDC40-1 and pDDC-4. pBR322 sequences are stippled while *Drosophila* sequences are nonstippled.



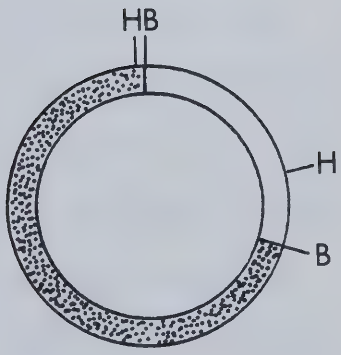
pDDC40-2



pDDC40-1



pDDC-1



pDDC-4

Various restriction digests of these 4 plasmids were analyzed on polyacrylamide gels. A total of 7 differences were detected between *Ddc*$^{+4}$ and Canton-S derived DNA. These differences are summarized in Figure 24 and Table 14. The Hind III site at coordinate 4.5 is missing from plasmid pDDC 40-2 (data not shown). This difference is called polymorphism 3 in Figure 24 and polymorphism c in Figure 20. Polymorphism 7 is a small decrease of less than 50 nucleotides in the size of the 1.1 Kb Bam HI/Hind III fragment (7.1 to 8.2). This difference can be seen in slots 5 through 9 of the 3.5% polyacrylamide gel displayed in Figure 25 and probably corresponds to polymorphism e as determined by Southern analysis (Figure 20).

The gel displayed in Figure 26 reveals several additional polymorphisms. Slots 1 through 6 are restrictions of pDDC 40-2 and pDDC-1 with Eco RI, Bam HI plus one of Ava I, Xba I, or Bgl II. In this case the third restriction enzyme is irrelevant. The alternating arrangement of these restrictions shows clearly that both the .6 Kb Eco RI/Bam HI fragment (5.7 to 6.3) and the .8 Kb Eco RI fragment (4.9 to 5.7) are slightly larger in pDDC 40-2. The difference in the Eco RI/Bam HI fragment is likely less than 20 nucleotides and is called polymorphism 6 in Figure 24. The polymorphism in the Eco RI fragment corresponds to polymorphism d as determined by Southerns (Figure 20). However if these plasmids are restricted by both Eco RI and Eco RV and electrophoresed on a polyacrylamide gel the difference in

Figure 24: The location of DNA polymorphisms identified by comparing cloned *Ddc*$^{+4}$ and Canton-S *Ddc* DNA. A total of seven differences numbered 1 to 7 were identified between cloned *Ddc*$^{+4}$ and Canton-S *Ddc* DNA. Their location is shown beneath the restriction map. A description of these polymorphisms is found in Table 14. The location of the *Ddc* transcription unit is indicated as in Figure 14.

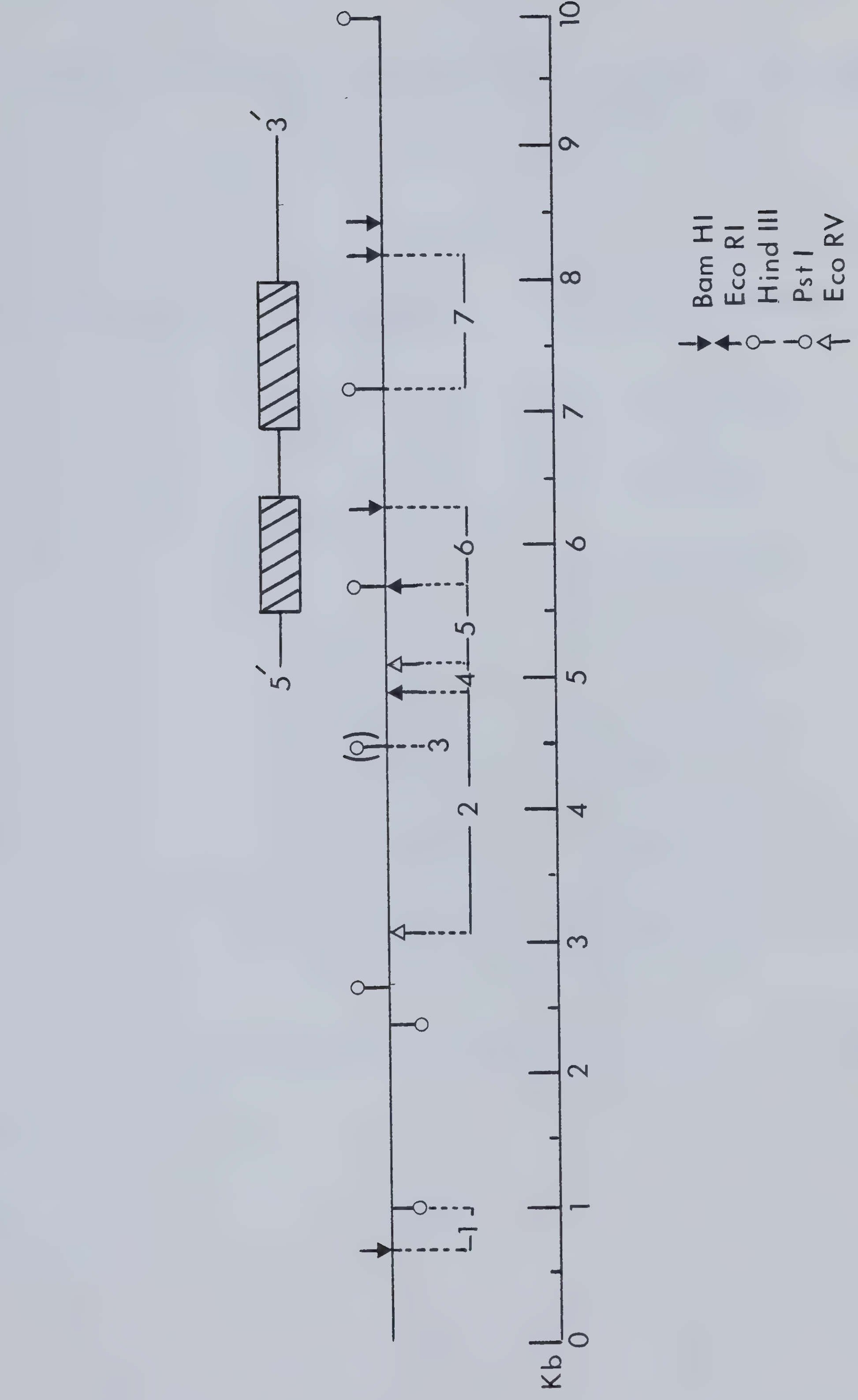

5´
3´
(○)
Kb
Bam HI
Eco RI
Hind III
Pst I
Eco RV

Table 14

Restriction Fragment Polymorphisms Revealed by Comparison of Cloned Canton-S and *Ddc*$^{+4}$ DNA

Polymorphism	Restriction Fragment	Change in Fragment Size
1	Bam HI/Pst I (0.7 - 1.0)	≃50 nucleotide increase
2	Eco RV/Eco RI (3.1 - 4.9)	≃50 nucleotide increase
3	–	loss of Hind III site at 4.5
4	Eco RI/Eco RV (4.9 - 5.1)	<20 nucleotide increase
5	Eco RV/Eco RI (5.1 - 5.7)	<20 nucleotide increase
6	Eco RI/Bam HI (5.7 - 6.3)	<20 nucleotide increase
7	Hind III/Bam HI (7.2 - 8.2)	<50 nucleotide increase

Figure 25: Restriction analysis of pDDC40-1 and pDDC-4. Approximately 1 μg/slot of plasmid DNA was restricted with endonuclease and electrophoresed on a 3.5% polyacrylamide gel. Slots 2, 4, 6, and 8 contain restricted pDDC40-1 DNA while slots 3, 5, 7, and 9 contain restricted pDDC-4 DNA. The DNA in slots 2 and 3 is Bam HI restricted; in slots 4 and 5 Hind III restricted; and the DNA in slots 6, 7, 8, and 9 is restricted with both Bam HI and Hind III. Slot 1 contains Taq I restricted pBR322.

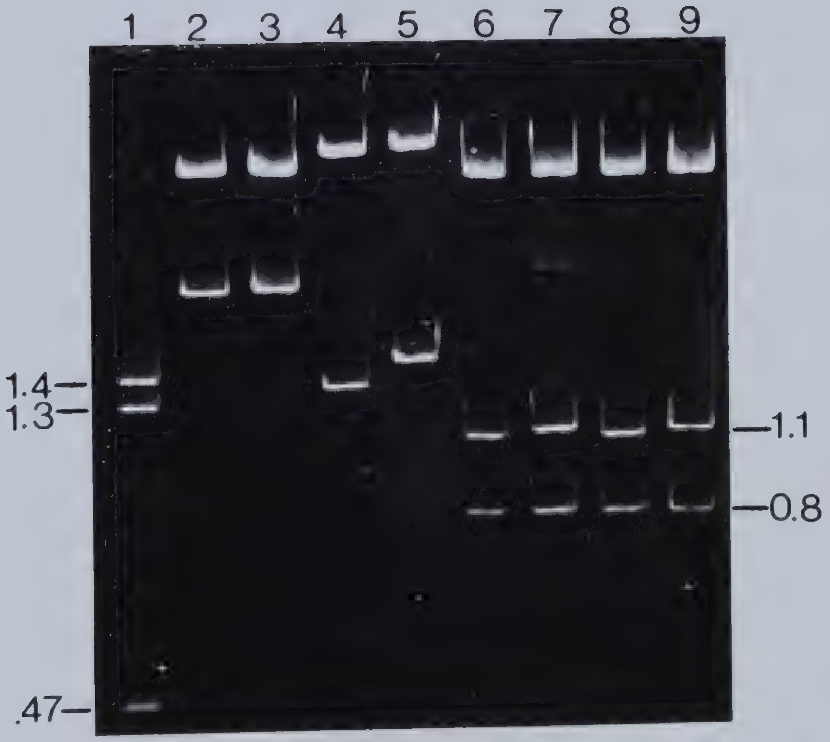

1
2
3
4
5
6
7
8
9
1.4
1.3
.47
1.1
0.8

Figure 26: Restriction analysis of pDDC40-2 and pDDC-1 (I). Approximately 1 μg/slot of plasmid DNA was restricted with endonuclease and electrophoresed on a 3.5% polyacrylamide gel. Slots 1, 3, and 5 contain pDDC40-2 DNA while slots 2, 4, and 6 contain pDDC-1 DNA. All DNA samples were restricted with Eco RI and Bam HI. In addition each DNA sample was restricted with a third enzyme: 1 and 2 with Ava I; 3 and 4 with Xba I; 5 and 6 with Bgl II.

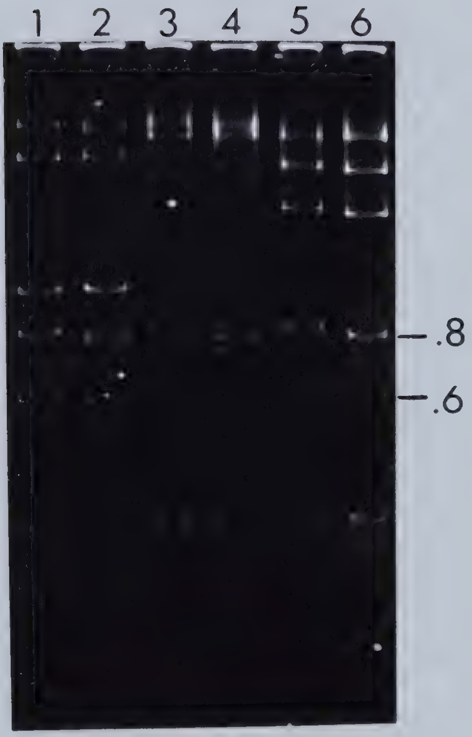
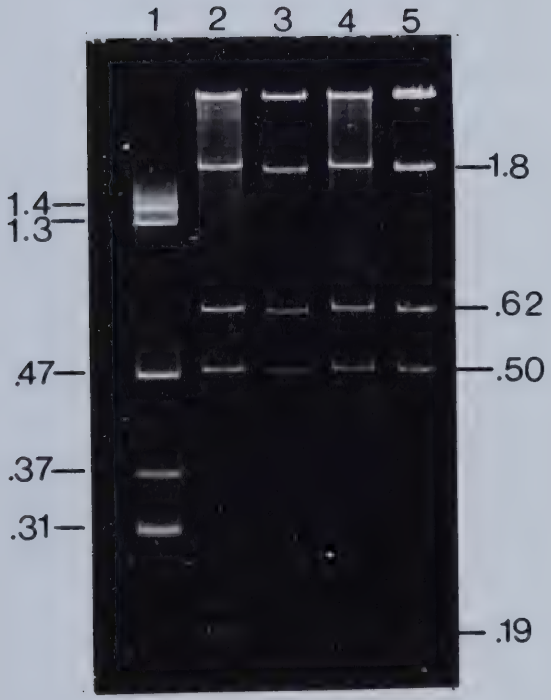

1
2
3
4
5
6
.8
.6

Figure 27: Restriction analysis of pDDC40-2 and pDDC-1 (II). Approximately 1 μg/slot of plasmid DNA was restricted with endonuclease and electrophoresed on a 3.5% polyacrylamide gel. Slots 2 and 4 contain restricted pDDC40-2 while slots 3 and 5 contain restricted pDDC-1 DNA. The DNA in slots 2 through 5 is restricted with Eco RI plus Eco RV. Slot 1 contains Taq I restricted pBR322.

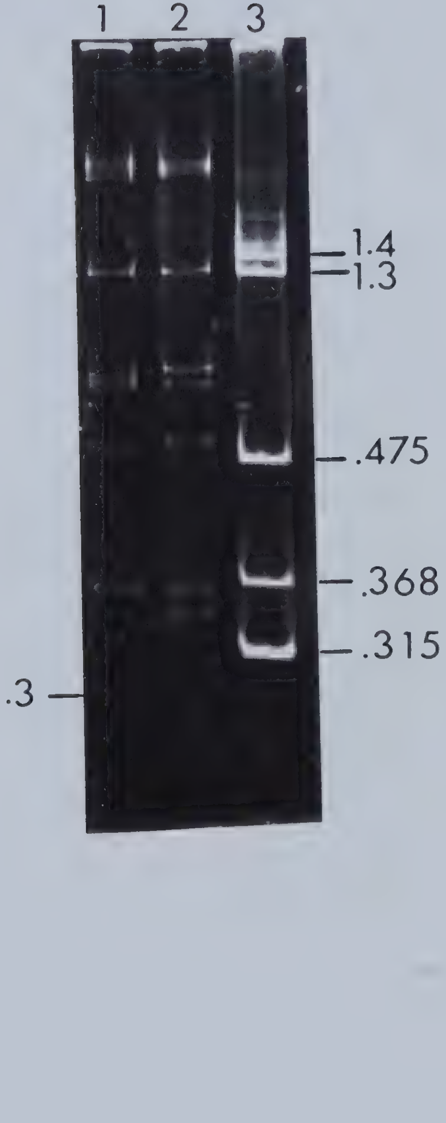

1
2
3
4
5
1.4
1.3
.47
.37
.31
1.8
.62
.50
.19

the size of the Eco RI fragment is found to be composed of 2 smaller size increases in the 2 Eco RI/Eco RV fragments (Figure 27). Polymorphism 5 is in the .5 Kb Eco RI/Eco RV fragment (5.2 to 5.7) and polymorphism 4 is in the .18 Kb Eco RI/Eco RV fragment (4.9 to 5.1). In slots 3 and 5 the .18 Kb Eco RI/Eco RV fragment is visible just below a .19 Kb fragment. This fragment is derived from pBR322 sequence. In slots 2 and 4, which contain *Ddc*$^{+4}$ DNA, the *Drosophila* fragment is comigrating with the pBR322 fragment because of its slightly larger size relative to the Canton-S version. Both of these small increases in size (polymorphisms 4 and 5) are probably less than 20 nucleotides. The gel in Figure 27 also illustrates polymorphism 2. The 1.8 Kb Eco RV/Eco RI fragment (3.1 to 4.9) is slightly larger in pDDC 40-2

Finally, the gel displayed in Figure 28 shows that there is a fairly substantial difference in the size of the .3 Kb Bam HI/Pst I fragment (0.7 to 1.0). The fragment from pDDC 40-2 is approximately 50 nucleotides larger than the analogous pDDC 1 fragment. This difference is called polymorphism 6 in Figure 24 and is probably the same as polymorphism a in Figure 20.

As stated above the differences observed when comparing cloned *Ddc*$^{+4}$ and Canton-S DNA are not identical to those seen when comparing *Ddc*$^{+4}$ and Canton-S DNA on genomic Southerns. The conflict involves the region of DNA between the Bgl II site at coordinate 2.7 and the Eco RI site at 4.9. Whole genome Southern analysis shows that the Bgl

Figure 28: Restriction analysis of pDDC40-2 and pDDC-1 (III). DNA samples were electrophoresed as in Figure 26. Slot 1 contains pDDC-1 DNA and slot 2 contains pDDC40-2 DNA. The DNA in both slots was restricted with Eco RI, Bam HI, and Pst I. Slot 3 contains Taq I restricted pBR322.


1
2
3
1.4
1.3
.475
.368
.315
.3

II/Eco RI fragment (2.7 to 4.9) is slightly smaller in the *Ddc*$^{+4}$*pr* strain (Figure 22). However analysis of pDDC 40-2 and pDDC-1 shows that the Eco RV/Eco RI fragment (3.1 to 4.9) derived from the *Ddc*$^{+4}$ clone is slightly larger than the analogous Canton-S fragment (Figure 27). The small Bgl II/Eco RV fragment (2.7 to 3.1) is identical in size in both plasmids (data not shown).

There are two possible explanations for this observation. It is possible that the polymorphism in the Eco RV/Eco RI fragment of pDDC 40-2 is rare in the *Ddc*$^{+4}$ population and that most *Ddc*$^{+4}$ genes have the smaller version of the fragment. Alternatively the Canton-S strain from which I isolated the DNA used for Southern analysis may have a larger Eco RV/Eco RI fragment than that Canton-S strain used to construct the original Canton-S library.

IV. Discussion

The study of gene regulation in eukaryotes has been hampered by the lack of mutants defective in regulatory processes. This is due in part to the inherent difficulties associated with the genetic manipulation of multicellular organisms. However other factors may contribute as well. For example, our ability to recover regulatory mutants from a mutagenized population depends, in a general way, on how stringent the requirement is for a specific DNA sequence. In prokaryotes single base changes have been shown to have dramatic effects on gene expression (Reznikoff and Abelson, 1978). Should we expect this to be true in eukaryotes as well? Regulation of gene expression may be less rigorous and less economical in eukaryotes. Because the complex organization of multicellular organisms buffers them from the environment, rapid and precise changes in gene expression are not required. Perhaps the complexity of eukaryotes permits a larger tolerance in regulatory processes. This might make it more difficult to perturb these processes by standard mutagenic procedures.

Natural populations provide an alternate source of regulatory variation. Several different studies have identified putative regulatory elements, both cis-acting and trans-acting, by analyzing naturally ocurring variation in enzyme activity (Bewley, 1981; McCarron et al; 1979; Paigen, 1978). This approach has been applied here to the dopa decarboxylase (DDC) gene-enzyme system.

A collection of wild type strains was surveyed for variation in DDC activity. Four strains representing the extremes of the range were selected for further analysis. My working hypothesis was that the observed variation in activity was due to changes in regulatory elements adjacent to the structural gene. This hypothesis was tested by asking two questions: 1) does the variation in enzyme activity map in the vicinity of the structural gene? 2) are the alterations in activity levels due to regulatory changes or structural changes in the enzyme? The phenotype of one regulatory variant, *Ddc*$^{+4}$, was investigated at several stages in development. Finally, the first step was taken towards the identification of DNA sequences responsible for the observed variation in gene regulation.

A. Survey of DDC activity

Newly eclosed adult flies from a total of 109 different strains were assayed for DDC activity. The range of activity among these strains was from 65% to 150% of Canton-S activity, or an approximately 2.5 fold range. If the two high outlying strains, WGM-40 and WGM-65, are excluded the range is slightly less than 2 fold. Because null mutations at the *Ddc* locus are recessive lethals, DDC is considered an essential enzyme (Wright et al., 1976). The enzyme's importance to the organism might be expected to impose greater limitations on the permitted range of activities in natural populations than other less essential enzymes.

Surprisingly, Laurie-Ahlberg et al. (1982) found variation of a magnitude similar to that found in this study for 22 different enzymes, including some nonessential (null-viable) enzymes, among 48 wild-type lines. The implications of these results are largely outside the scope of this work. It is apparent however, that the degree of evolutionary constraint on the accumulation of variation in enzyme activity cannot be directly extrapolated from the severity of mutant phenotype in the laboratory. In any case, it appears that even for an essential enzyme such as DDC, natural populations contain enough activity variation to be potentially useful in the study of gene regulation.

B. Genetic analysis of activity variant strains

Strains WGM-06, -19, -40, and -65 were selected for further study because they fall at, or near, the extremes of the range of activities (Figure 6). The object of the genetic analysis performed in this study was to determine if the element(s) responsible for the activity variation maps in the region of the structural gene for DDC. Wright et al. (1976) place the gene for DDC at 54.1 on the left arm of the second chromosome.

Laurie-Ahlberg et al. (1982), in their study of variation in activity for 23 enzymes in *Drosophila*, showed that chromosomes other than the chromosome which carries the structural gene for an enzyme, were a significant source of variation. As a consequence, the autosomal segregation

pattern of the activity variation was examined for the four selected strains. The results presented in Table 4 demonstrate that for each of strains WGM-06, -40, and -65, activity differences segregated with the second chromosome. Strain WGM-19 had only a slight activity difference associated with the second chromosome and was therefore dropped from this study.

If the data in Table 3 and Table 4 are compared, it is apparent that the DDC overproduction in the +/+ ; +/Sb progeny of the crosses involving WGM-40 and WGM-65 is more extreme than in the parent strain. This kind of observation was made several times during the course of this work. Outcrossing increased the DDC activity of the variant strains but did not increase the activity of those strains with a level of activity closer to the median of the range. Modifier loci may exist which act to moderate DDC activity levels towards some optimum level, presumably the median of the distribution presented in Figure 6.

The structural gene for DDC is flanked by the visible markers *hk* at 53.9 and *pr* at 54.5 (Wright et al., 1976). Crosses were performed with WGM-06, -40, and -65 to test whether any activity variation mapped to this region. Briefly, the scheme involved selecting recombinant chromosomes from + +/*hk pr* flies, where the source of the hk^{+} pr^{+} chromosome was one of the activity variant strains. The recombinant chromosomes, both + *hk* and *pr* + were made homozygous and the segregation of the element(s) responsible

for activity variation determined by DDC assays (Figure 2). Flies homozygous for recombinant chromosomes derived from strains WGM-06 and WGM-65 did not survive. The cause of this lethality was not investigated further, but it seems likely that both strains had accumulated a number of recessive lethal mutations on the second chromosome since the time of isolation.

Strain WGM-40 did not present the same problem. A total of 23 strains homozygous for second chromosomes generated by recombination between alleles of *hk* and *pr* were recovered from this scheme (Tables 5 and 6). DDC activity determinations showed that the activity variation observed in WGM-40 was segregating as a single unit. The element responsible for the variation was named Ddc^{+4}. However the data do not unambiguously place Ddc^{+4} between *hk* and *pr*, as was first thought. The problem lies in determining whether Ddc^{+4} lies to the right or to the left of *hk*. Only 2 of 23 recombinants were generated by a crossover event which separated the *hk* allele and the Ddc^{+4} element. This implies either that Ddc^{+4} is between *hk* and *pr* but much closer to *hk*, or that Ddc^{+4} lies to the left of *hk*. If this second map position were correct, a double crossover consisting of one event between Ddc^{+4} and *hk*, and one event between *hk* and *pr* would generate the two recombinants mentioned above. Assuming no interference, it is possible to calculate the approximate map position of Ddc^{+4} if it were to map to the left of *hk*. The putative double event was recovered twice

while recombinants generated by a single crossover between *hk* and *pr* were recovered 21 times. The map distance between *hk* and *pr* is .6 map units. Therefore the frequency of the double event is 2/21 X (.006) = .00057. This frequency is composed of the frequency of an event between *hk* and *pr* (.006) times the frequency of an event between *hk* and *Ddc*$^{+4}$ (y). Solving the equation .00057 = (.006)(y) gives y = .093, meaning that *Ddc*$^{+4}$ is located 9.3 map units to the left of *hk*. Experiments designed to distinguish between the 2 possible map positions are currently underway.

C. Developmental characterization of *Ddc*$^{+4}$

Approximately 90% of DDC activity in *D. melanogaster* is localized in the epidermal cells. The enzyme's role in these cells is to provide precursors for the process of cuticle sclerotization, which occurs after every ecdysis and at pupariation. Regulation of the gene for DDC is therefore precisely and inextricably linked to the developmental program of the organism. This is illustrated in Figure 5. Sharp peaks of enzyme activity occur at hatch, the two larval molts, pupariation, and eclosion. An additional peak of activity has been detected at pupation (Wright et al., 1982). Initial interest in the DDC gene-enzyme system focussed on the peak of activity at pupariation. Numerous studies in our laboratory and others (Clark and Hodgetts, unpublished results; Fragoulis and Sekeris, 1975b; Kraminsky et al., 1980) support the view that the increase in DDC

activity at this stage is related to the increase in the insect steroid hormone ecdysterone, or molting hormone (Figure 5). In this way expression of the gene for DDC is conveniently linked to the larger developmental program of pupariation. However, at other stages, ecdysterone appears to play no direct role in the regulation of the *Ddc* gene, even though the gene is under rigorous developmental control at these times as well. The peaks of enzyme activity at hatch, the larval molts, and eclosion follow peaks of ecdysterone titer by at least 10 hours and in fact occur at times when ecdysterone titer is at the basal level. If ecdysterone is interacting directly at the DNA level to induce expression of the *Ddc* gene at pupariation, then there must be additional regulatory components active at the other times when the gene is expressed.

The level of DDC activity in a *Ddc*$^{+4}$ strain was compared to the level of activity in Canton-S at hatch, the 2/3 molt, pupariation, and eclosion. Table 7 shows that *Ddc*$^{+4}$ had more DDC activity at all stages except pupariation. At this stage the variant had only half the Canton-S level. Mapping data (Table 8) indicates that the variation in activity at pupariation is behaving the same genetically as the overproduction at eclosion, implying that either the same element is responsible for the 2 opposite activity alterations or that 2 closely linked elements are involved.

If we assume that both effects are due to the same alteration, several points can be made. It is difficult to imagine a structural change in the enzyme that would result in an increase in activity at one stage and a decrease at another stage. Similarly, a simple change in a basic promoter sequence, such as a T-A-T-A box, would not result in opposite effects at two different stages. Instead, *Ddc*$^{+4}$ may define a complex regulatory element which interacts with a hormonal stimulus at pupariation, and some undefined effector at other times of *Ddc* gene expression.

Besides epidermal cells, DDC activity appears in nervous tissue (Livingstone and Tempel, 1983) and in ovaries (Wright et al., 1982). Experiments are underway to determine the effects of *Ddc*$^{+4}$ on gene expression in these tissues.

D. The regulatory nature of *Ddc*$^{+4}$

The developmental characterization described above suggests that the activity variation defined by *Ddc*$^{+4}$ is due to changes in regulation and not changes in enzyme structure. Further support for this hypothesis was obtained in three ways. First, measurement of CRM levels by immunotitration with a monospecific antiserum showed that changes in enzyme activity coincided with changes in CRM. Second, analysis of DDC from *Ddc*$^{+4}$ and Canton-S on two different gel systems failed to detect any structural differences. Third, comparison of the steady state level of DDC mRNA in *Ddc*$^{+4}$ and Canton-S strains shows that

differences in activity levels are reflected by differences in the amount of DDC transcript.

CRM levels in *Ddc*$^{+4}$*pr* and Canton-S strains

Immunotitration of DDC with a monospecific antiserum was done on crude homogenates of white puparia and young adults. In both stages the ratio of *Ddc*$^{+4}$:Canton-S CRM was very close to the ratio of *Ddc*$^{+4}$:Canton-S enzyme activity (Tables 7 and 9). These results imply that activity differences between *Ddc*$^{+4}$ and Canton-S are due to changes in the rate of synthesis of enzyme or in the stability of enzyme. In addition, differences in CRM between stages of the same strain were mirrored by the same relative differences in enzyme activity, eliminating the possibility that stage to stage modulations are due to some undescribed cytoplasmic factor (Figure 11). There is no evidence that enzyme activity is regulated by anything other than the amount of enzyme present in the organism.

Electrophoretic analysis of DDC

DDC from *Ddc*$^{+4}$ and Canton-S strains was analyzed on two gel systems in an attempt to detect structural differences. The SDS polyacrylamide gel system resolves differences in size while the cellulose acetate system separates on the basis of both size and charge. No difference in migration between the two enzymes was observed in either system (Figures 15 and 16). However, this analysis is not

particularly rigorous and does not exclude the possibility that small structural differences exist.

DDC transcript levels in *Ddc*$^{+4}$ and Canton-S strains

The best way to demonstrate gene regulation at the level of transcription is to pulse label nuclear RNA with radioactive nucleotides and measure the amount of incorporation into a specific transcript by hybridization with a DNA probe. Unfortunately, the technical difficulties associated with such an approach for the *Ddc* gene in *Drosophila* are rather formidable. In lieu of a direct measurement of transcriptional activity, the steady state level of DDC transcript was compared in *Ddc*$^{+4}$ and Canton-S strains by examination of northern blots. No attempt was made to determine the actual concentration of DDC transcript in these experiments. The object was to compare the levels of poly-A^{+} transcript in these two strains and relate that comparison to relative levels of enzyme activity and CRM.

In both newly hatched first instar larvae and newly eclosed adults there is between 40% and 50% more DDC transcript in *Ddc*$^{+4}$ than in Canton-S, while at pupariation, *Ddc*$^{+4}$ has 50% less DDC transcript (Table 12). In light of this result, the differences in CRM level described above must be due to differences in the rate of enzyme synthesis. This difference, in turn is a consequence of an altered concentration of DDC transcript. The question now is: what causes the alteration in the size of the DDC transcript

pool? One possibility is certainly a change in the rate of transcription, but other alternatives cannot be ignored. For example, in *Dictyostelium* the stability of some mRNA species changes as development proceeds (Chung et al., 1981). In humans, a mutant β-globin gene has been identified that produces a very unstable mRNA (Maquat et al., 1981). Other steps in RNA processing such as intron splicing, polyadenylation, and transport may also be affected.

E. Sensitivity to dietary α-methyl dopa

The dopa analogue α-methyl dopa (α-MD) has been shown to be an inhibitor *in vitro* of both mammalian (Lovenberg et al., 1962) and *Drosophila* (Sparrow and Wright, 1974) dopa decarboxylase. Supplementing *Drosophila* food with α-MD at concentrations of approximately 10^{-4} M causes lethality in larval stages. The larvae die shortly after the larval molt suggesting that lethality is caused by the inhibition of DDC and the consequent inability to sclerotize newly deposited cuticle. Sparrow and Wright (1974) and Sherald and Wright (1974) attempted to use α-MD supplementation as a means of selecting for mutants with decreased and increased levels of DDC activity, believing that such changes would be reflected by changes in sensitivity to the drug. Surprisingly, they found that there was not a simple relationship between α-MD sensitivity and DDC activity. A total of 33 α-MD sensitive mutants have now been isolated (Wright et al., 1982), all alleles of the *l(2)amd* gene. These mutations have only small

affects on DDC activity. Conversely, null mutations at the *Ddc* locus do not effect sensitivity to α-MD.

Despite all this, α-MD sensitivity does seem to be related to the amount of DDC activity in some way. Two strains of *Drosophila* have been described which overproduce DDC. Both of these strains are also less sensitive to α-MD (Marsh and Wright, 1979; Sherald and Wright, 1974). As can be seen in Figure 8, *Ddc*$^{+4}$ behaves in a similar way. Is the change in α-MD sensitivity due to increased DDC activity? By using deficiencies and duplications of the *Ddc* region, Marsh and Wright constructed strains with different doses of the *l(2)amd* and the *Ddc* genes. They found that as the relative dose (*Ddc*$^{+}$:*amd*) increased the sensitivity to α-MD also increased (personal communication). This result suggests that the decreased sensitivity in overproducer strains is due to something other than a simple increase in DDC activity. Marsh and Wright (personal communication) have proposed that the dual DDC/α-MD phenotype in these strains may be due to changes in control sequences shared by the *l(2)amd* and the *Ddc* genes. Alternatively, the complexity of DDC/α-MD interactions may reflect undescribed complexity in monoamine metabolism.

F. Molecular analysis of the *Ddc* gene in *Ddc*$^{+4}$

The molecular studies of the gene for DDC described here, were facilitated by the cloning work of Hirsh and Davidson (1981). By using cDNA probes prepared from third

instar larvae and early embryos, stages when the *Ddc* gene is expressed, and not expressed respectively, they were able to recover several recombinant λ carrying the *Ddc* gene from a *Drosophila* library. The library was constructed in λ Charon-4A by Maniatis et al. (1978) using *Drosophila* DNA from a Canton-S strain. The recombinant λ which carry *Ddc* sequences were kindly supplied to us by J. Hirsh and used in our laboratory as a source of *Ddc* specific probes.

The goal of this section of this study was to compare in some detail the DNA organization of the *Ddc* gene and surrounding region, in *Ddc*$^{+4}$ and Canton-S strains, in order to identify differences which may be responsible for regulatory variation. Restriction enzymes were used to examine Canton-S and *Ddc*$^{+4}$ sequences for differences in the pattern of restriction sites and the size of restriction fragments. Single base changes go unnoticed with this kind of analysis unless they happen to disrupt a restriction site. However evidence from several studies suggests that differences detectable by restriction analysis should not be unexpected.

In those instances where mutants with increased rates of transcription have been analyzed at the molecular level, large scale rearrangements have been found 5' to the gene. Sherman et al. (1981) found that mutants in yeast which overproduce iso-2-cytochrome c carried translocations, insertions, and deletions in the 5' flanking region. A result reported by Scherer et al. (1982) on the *his-3* gene

in yeast is even more bizarre. They constructed *in vitro*, a *his-3* gene completely lacking 5' flanking sequence. This gene was transcriptionally inactive. The mutant gene was introduced into yeast cells by transformation. They then selected for events which allowed transcription of the gene by growing the transformed cells on medium lacking histidine. Revertants fell into 2 classes. One class all contained insertions of the yeast transposon *Ty1* 5' to the gene. *Ty1* elements have also been shown to act as promoters when inserted adjacent to the alcohol dehydrogenase gene (Williamson et al., 1981). The second class of revertants had all experienced duplication of sequences 5' to the *his-3* gene. Most of the duplications involved pBR322 and λ sequences which do not normally possess promoter activity. Apparently rearrangement of these sequences activated normally silent promoters in the prokaryotic DNA.

In *Drosophila*, many mutations have been shown to be due to large scale rearrangements of DNA sequence. Several mutant alleles of the white locus carry insertions of what appear to be transposable sequences (Collins and Rubin, 1982; Zachar and Bingham, 1982). Another white mutation, called *white-ivory*, is associated with a 2.9 Kb tandem duplication within the white locus (Karess and Rubin, 1982). Of particular interest is a mutation called *Dominant zeste-like*. This mutation seems to be caused by an insertion of a 13 Kb fragment of DNA at the extreme right end of the white locus. On the basis of genetic evidence and the

molecular analysis of a collection of white alleles, Zachar and Bingham (1982) suggest that this element acts at a distance to suppress expression of the white gene. There is no conclusive evidence as yet that any of these insertions and rearrangements in *Drosophila* cause regulatory changes, but the studies in yeast discussed above suggest that such evidence will be forthcoming.

Cloning the *Ddc* gene from a *Ddc*$^{+4}$ strain

The bacteriophage λ 1059 was chosen as the vector for construction of a *Drosophila* library for two major reasons. First, and most important, because λ 1059 accepts DNA inserts with Sau 3A restricted ends, it is easy to construct a library containing a random collection of DNA fragments by partial restriction of the insert DNA with Sau 3A. Second, since it is possible to select directly for recombinant phage, there is no need to purify λ arms before library construction. Unfortunately, λ 1059 is a prototype model and consequently has several annoying disadvantages. It was originally constructed by a complex recombination event involving 2 different phages and a pBR322 derived plasmid containing λ sequence including the λ attachment site (Brenner et al.,1982). The product (Figure 3) is a phage with duplicated attachment sites flanking a central region containing both λ and pBR322 sequences. This central region also contains the *red* and the *gam* genes. These two genes are required for positive selection of recombinant phage. A

phage which is *red*$^+$ *gam*$^+$ is said to have a *spi*$^+$ phenotype and cannot grow on a host lysogenic for phage P2. If the central fragment is replaced with foreign DNA the phage becomes *spi*$^-$ and will grow on a P2 lysogen. However, according to Maniatis (1982), *int* gene mediated recombination can also generate *spi*$^-$ phage capable of growing on a P2 lysogen. These phage appear at a frequency of 10^{-3}. This contamination would not be a serious problem if the central fragment did not contain pBR322 sequence. It is common practise to maintain probe sequences in pBR322 derived plasmids. Even if the DNA fragment to be used as a probe is gel purified, some pBR322 sequence will inevitably remain. When this probe is used to screen a library constructed in λ1059, all those *spi*$^-$ phage generated by the *int* mediated recombination event will score positive because of pBR322 homology. A final inconvenience associated with λ1059 was encountered when analyzing recombinant phage recovered from the library. Each recombinant λ was constructed by ligation of a *Drosophila* Sau 3A fragment with 2 λ arms, each with a Bam HI terminus. This ligation event destroys the Bam HI restriction site, making it impossible to separate insert DNA from vector DNA by restriction. As a consequence it was difficult to determine the exact endpoints of the inserted *Drosophila* fragment.

When the *Drosophila* library constructed with DNA from a *Ddc*$^{+4}$ strain was screened with gel purified 1.9 Kb Bam HI fragment (6.3 to 8.2, Figure 14), a very large number of

plaques scored faintly positive. These were probably *spi*⁻ λ1059 hybridizing to contaminating pBR322 sequences in the probe. Standing out above this background were 7 strongly positive plaques. As described in the Results, only 2 of these 7 phage contained inserts with a restriction pattern similar to that of the *Ddc* region. These 2 phage (λ4 and λ15) were identical and were probably clonally derived from the same ligation product. The other 5 phage formed 2 groups. One group contained 3 identical phage (λ8, λ12, and λ13), again probably clonally derived. Although λ8 was shown to contain DNA from the *Ddc* region by Southern blotting (data not shown), restriction of this DNA did not generate any familiar restriction fragments. These phage may contain a rearranged copy of the *Ddc* region which could be present in the *Ddc*$^{+4}$ population. If present, this rearrangement would have to be rare since Southern analysis with the same DNA used to construct the library did not reveal any unexpected restriction fragments. Alternatively these phage may have been generated by ligation of 2 *Drosophila* fragments, one from the *Ddc* region and one from some other location in the genome, with the 2 λ arms. If the fragment from the *Ddc* region was small enough it may be unidentifiable by the restriction analysis performed.

The second group of phage had 2 identical members (λ5 and λ9). No attempt was made to confirm homology between their insert DNA and DNA in the *Ddc* region by Southern blotting. Restriction analysis suggested that these phage

had undergone a rearrangement which introduced an Eco RI site into the left arm. An *int* mediated recombination event may have been responsible for this rearrangement.

Restriction analysis of the *Ddc* region from *Ddc*$^{+4}$

The *Ddc* region of *Ddc*$^{+4}$ was compared to that of Canton-S by examination of both genomic Southern blots and cloned sequences carried on bacterial plasmids. No large rearrangements of DNA sequence were detected . *Ddc*$^{+4}$ lacks a Hind III site which is present in Canton-S DNA at coordinate 4.5 (Figure 14). This polymorphism is also present in several other strains of *Drosophila melanogaster* (Hirsh, personal communication), including strains carrying the CyO balancer chromosome (Figure 21). A number of restriction fragments were found to be slightly different in size in the two strains (Tables 20 and 24). These differences are all less than 50 nucleotides and lie in 5' flanking regions as well as transcribed regions of the *Ddc* gene. For the purposes of discussion these differences will be called insertions and deletions. However, without DNA sequence information, we cannot be sure that each size change is due to a single insertion or deletion event.

It is important at this point to compare the results obtained through genomic Southern analysis and examination of cloned sequences. When DNA from a *Ddc*$^{+4}$ strain was analyzed on Southern blots, only one pattern of restriction fragments was observed, suggesting a high degree of

homogeneity in the *Ddc* region in the population of *Ddc*$^{+4}$ flies used as a source of DNA. This implies that any differences detected on Southerns must be present at a high frequency. On the other hand differences detected in a single copy of the region, isolated and propagated by cloning, need not be present at a high frequency in the population. A small amount of polymorphism, even in a recently isogenized strain, is probably inevitable. There are two ways of ensuring that a cloned *Ddc* region is typical of the population as a whole. One way is to recover a reasonable number of overlapping clones. Unfortunately only one phage containing *Ddc* sequence was recovered in this study. The second way is to show that differences observed on cloned sequences can also be detected on genomic Southerns. This comparison is possible here. Of the 7 differences between *Ddc*$^{+4}$ and Canton-S observed in cloned sequences, 6 are also observed on genomic Southerns. Conversely all but one of the differences detected on genomic Southern blots also appear in the cloned sequences. The one inconsistency involves the region of DNA between coordinate 2.7 and 4.9 in Figure 14. Genomic Southerns show that *Ddc*$^{+4}$ carries a small deletion in this region relative to Canton-S, whereas plasmid pDDC 40-2 has a small insertion in this same region when compared to pDDC-1. This means that either the *Ddc*$^{+4}$ DNA carried in pDDC40-2 is not typical of the *Ddc*$^{+4}$ strain or that the Canton-S clone in pDDC-1 does not contain the version of this region common in the

Canton-S strain. It is impossible to determine which of these 2 alternatives are correct without further experiments. However it is worth noting that the same sample of *Ddc*$^{+4}$ DNA was used for both genomic Southerns and library construction. The same is not true for Canton-S since the Canton-S library was constructed before 1978 (Maniatis et al., 1978).

It is difficult to be precise about the position of each insertion and deletion in relation to the *Ddc* gene because of uncertainty concerning the exact dimensions and complexity of the *Ddc* transcription unit. Early work on cloned *Ddc* sequences indicated that the *Ddc* gene consisted of 3 exons and 2 introns (Hirsh and Davidson, 1981; Hirsh, personal communication) as drawn in Figure 14. However recently it has become clear that the pattern of transcription, and the pattern of transcript processing are not simple. Gietz and Hodgetts (manuscript in preparation) find several transcripts of sizes not predicted by simple removal of the two large introns depicted in Figure 14. In addition they find that some *Ddc* transcripts are stage specific. It appears that differential promoter selection and/or differential splicing may be involved in *Ddc* gene expression. Until DNA sequencing and accurate transcript mapping are completed, we will not know exactly which sequences are transcribed, nor which transcribed sequences code for DDC.

Despite this uncertainty, the information in hand does allow one to make some general statements about the location of the 6 changes. Denaturing polyacrylamide gel electrophoresis shows that there is no difference in the size of the *Ddc*$^{+4}$ and Canton-S enzymes (Figure 12). This implies that none of the insertions or deletions occur in coding regions of the gene. Referring to Figure 24, differences 6 and 7 must therefore fall in transcribed but noncoding regions of the gene. Differences 4 and 5 may fall either in transcribed sequences or in flanking sequences depending on where the exact site of transcription initiation is. Polymorphisms 1 through 3 (Figure 24) and polymorphism b (Figure 20) all lie in 5' flanking regions.

What effects do each of these differences have on gene expression? Specific information pertaining to this question exists for polymorphisms 1 and 3 only. Several chromosomes, including the *CyO* balancer chromosome, lack the Hind III site at coordinate 4.5 (polymorphism 3, Figure 24). Strains carrying a *CyO* chromosome have a level of DDC activity similar to that of Canton-S (data not shown) suggesting that this change is neutral with respect to *Ddc* gene expression. The same logic can be applied to polymorphism 1. Southern analysis shows that both *Ddc*$^{+4}$ and the *CyO* chromosome carry an insertion of DNA in the .3 Kb Bam HI/Pst I fragment (.7 to 1.0). Of course without sequence information it is impossible to say that these two insertions are the same. However it is clear that DNA sequence variation in this

region need not affect gene expression. Recent experiments by Scholnick et al. (1983) also address this point. They have been applying recently developed DNA mediated transformation techniques in *Drosophila* to the DDC system and find that transformation with a DNA fragment including the *Ddc* gene and 5' flanking sequence up to the Pst I site at coordinate 2.4 will rescue flies homozygous for the *Ddc*^{ts2} mutation. This DNA fragment lacks the region containing polymorphism 1. Five different transformants, each with the *Ddc* gene integrated at a different chromosomal location, were recovered. Four of these five had approximately 30% more activity at eclosion than the Canton-S strain. In addition one of these four had decreased activity at pupariation. The level of activity at pupariation in the other transformants was not reported. The similarity between these transformants and *Ddc*$^{+4}$ is striking. Scholnick et al. (1983) suggest that the enzyme activity differences they observed may be due to the lack of regulatory sequences not included on the DNA fragment used for transformation. Such regulatory sequences, if they exist, may be in the region of polymorphism 1.

Polymorphisms 4 and 5 (Figure 24) and polymorphism b (Figure 20) all probably lie in 5' flanking regions where regulatory sequences have been found in studies on other eukaryotic genes. If one of these differences is responsible for the *Ddc*$^{+4}$ phenotype its effect would likely be on transcription. Muskavitch and Hogness (1982) found that in

Drosophila the presence of small naturally occurring deletions between 300 and 500 nucleotides upstream from the *Sgs-4* gene correlate with changes in the expression of that gene. Further experimentation is required before any correlations can be established between the insertions 5' to the *Ddc* gene and changes in gene expression.

Polymorphism 6 and 7 both lie in transcribed but noncoding regions of the *Ddc* gene. Again, until more experiments are performed, we can only speculate about the effects these changes may have on gene expression. Numerous mutant human globin genes have been shown to contain sequence changes within introns which alter normal splicing patterns. Analysis of three such mutant β-globin genes by Triesman et al., (1983) showed that inactivation of a 5' splicing signal leads to the use of normally silent splicing signals. The result was a decrease in normal mRNA and the appearance of altered processing products. A fourth mutant had experienced a nucleotide change which created a 5' splicing signal in the middle of an intron and caused production of an RNA species with an extra exon. It is conceivable that one of changes 6 or 7 are causing similar disturbances in splicing. Although no aberrant *Ddc* transcripts have been observed in *Ddc*$^{+4}$ RNA, the experiments performed to date were not designed to detect any unusual RNAs. An incorrectly processed molecule, incapable of being translated, may differ in size from the normal mRNA by only a few nucleotides. Alternatively an incorrectly processed

transcript may be unstable and therefore not appear on northern blots at all.

Recent studies on immunoglobulin genes suggest that intron sequences may play a more active role in gene regulation as well. Parslow and Granner (1982), investigating expression of the kappa light chain immunoglobulin gene found that expression of this gene is associated with a DNase I hypersensitive site located within an intervening sequence. DNase I hypersensitive sites are commonly found in 5' flanking regions of actively expressed genes (Elgin, 1981). At a recent conference (Boss, 1983), both David Baltimore and Susumu Tonegawa presented evidence suggesting that sequences similar to the SV40 enhancer lie within the J-C intron of both the kappa light chain and the heavy chain immunoglobulin genes. They also report that these sequences are necessary for transcription of the two genes.

G. Polymorphism in the *Ddc* region

Restriction analysis of DNA in the *Ddc* region in Canton-S and *Ddc*$^{+4}$ strains has revealed a surprising number of small differences in restriction fragment size. These results are unique among those obtained for the single copy sequences examined to date. For example studies on the human globin genes identified several restriction site polymorphisms (Orkin et al., 1982). However, the widespread occurrence of small deletions and insertions was not

reported. This may be because the resolution of the techniques used was not sufficient to detect such small differences. In *Drosophila*, Muskavitch and Hogness (1982) have examined the DNA organization 5' to the *Sgs-4* gene in nine different strains. They found that three strains had the same deletion of about 50 nucleotides approximately 500 nucleotides upstream from the transcription start site. Another strain had a 100 nucleotide deletion in the same region. It should be noted that the four strains carrying deletions were selected for study because they express the *Sgs-4* gene at reduced levels.

Although it is difficult to place the results described here on the *Ddc* gene in any kind of larger context in terms of kind or amount of polymorphism, it is clear that when dealing with wild-type strains a certain amount of neutral DNA sequence variation should be expected. The use of such wild-type strains as a source of variation will of necessity involve determining which changes are meaningless and which are responsible for the phenotype under study.

H. Concluding remarks

A genetic element causing stage specific changes in the expression of the *Ddc* gene has been described. This element, named *Ddc*$^{+4}$, probably lies in the immediate vicinity of the structural gene for DDC and alters the amount of enzyme activity by changing the size of the DDC mRNA pools. Examination of DNA sequences in the *Ddc* region identified 7

sequence differences between *Ddc*$^{+4}$ and Canton-S strains, one or more of which may be responsible for the regulatory changes.

The task of sorting out the effects of each of the polymorphisms can be approached in several ways. First of all DNA sequence information for both Canton-S and *Ddc*$^{+4}$ strains as well as a better understanding of the source and fate of the various DDC transcripts may provide some healthy clues. For example, perhaps one of the insertions lies adjacent to a recognized regulatory sequence such as a T-A-T-A box. Any perturbation in the first 100 nucleotides upstream from the transcription start would be suspect since this region has been shown to contain critical regulatory sequences in other systems. It would also be interesting to know what the pattern of DNase I hypersensitive sites is around the *Ddc* gene, and where the DNA sequence differences lie in relation to those sites.

The direct determination of the effects of each sequence difference on gene expression can only be accomplished by examination of each polymorphism individually in a Canton-S background. To do this genetically would be incredibly tedious at the least, and perhaps impossible. Luckily, it has recently become possible to introduce *in vitro* gene constructs into the *Drosophila* genome by DNA-mediated transformation (Rubin and Spradling, 1982). The DNA of interest is integrated into the genome after it is injected into early embryos on a plasmid

carrying a particular kind of *Drosophila* transposable element called a *P* element. As mentioned earlier, these manipulations have been performed in the laboratory of J. Hirsh (Scholnick et al., 1983) with the *Ddc* gene. They analyzed a number of transformants. In each transformatant the *Ddc* gene had integrated at a different chromosomal location and was expressed at near normal levels. Using this technology it should be possible to determine which of the DNA polymorphisms associated with *Ddc*$^{+4}$ is responsible for the regulatory variation by constructing recombinant *Ddc* genes *in vitro* and examining their expression *in vivo* by transformation.

It will not be possible to construct a complete picture of *Ddc* gene regulation by studying one activity variant. Many more strains need to be examined. The analysis of two such strains (WGM-06 and WGM-65) was abandoned in this study because of early problems with their genetic characterization. Now that recombinant DNA technology has reached a high degree of sophistication, and in particular, now that practical *Drosophila* transformation systems are available it should be possible to examine cis-acting regulation in these strains without doing conventional genetics.

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